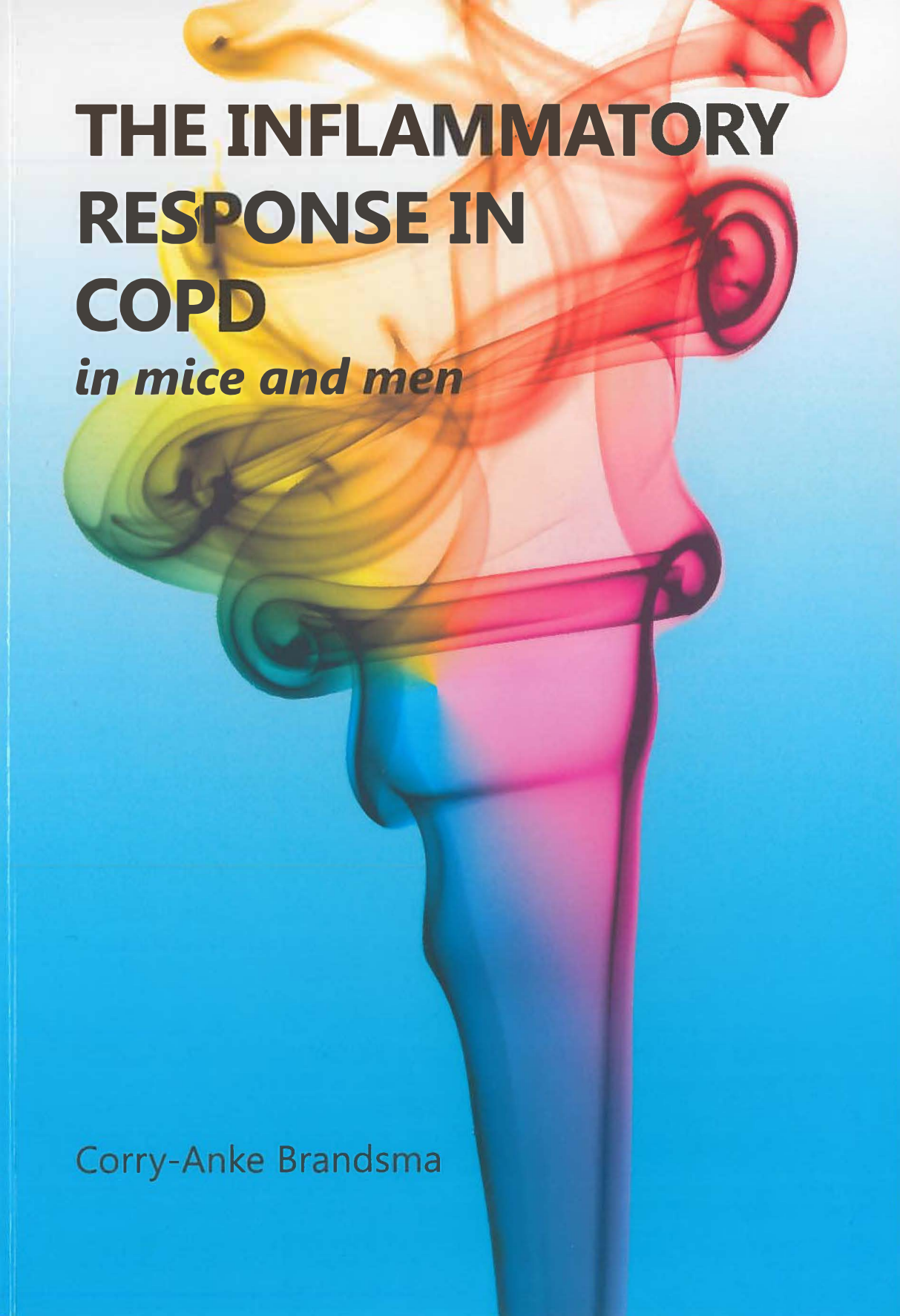
The inflammatory response in COPD in mice and men

Brandsma, Corry Anke

THE INFLAMMATORY RESPONSE IN COPD

in mice and men



Corry-Anke Brandsma

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Stellingen behorende bij het proefschrift

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1. Het feit dat kortdurend roken in muizen ontstekingsremmende effecten heeft, is geen adequaat excuus voor COPD patiënten om te (blijven) roken (dit proefschrift).

2. Blootstelling aan stikstofdioxide versterkt de rook-geïnduceerde ontstekingsreactie in muizen niet, maar draagt wel bij aan de gevoeligheid voor infecties.

3. Roken veroorzaakt een antigeen specifieke immuunrespons (dit proefschrift).

4. Het is zeer aannemelijk dat de rook-geïnduceerde specifieke immuunrespons bijdraagt aan de pathogenese van COPD.

5. Om de rol van regulatoire T cellen in COPD te onderzoeken, moet de functionaliteit van deze cellen specifiek in longweefsel bestudeerd worden.

6. HO-1 inductie zorgt via een toename van regulatoire T cellen voor een verminderd aantal rook-geïnduceerde B-cel-infiltraten in muizen (dit proefschrift).

7. Een rokende muis raakt weliswaar verslaafd, maar zal nooit volledig COPD ontwikkelen.

8. Gezonde rokers bestaan niet.

9. Een 'gezonde' roker onderwerpen aan longfunctieonderzoek voor een wetenschappelijke studie leidt tot het risico op voortzetting van diens schadelijke rookgedrag.

10. Het feit dat tegenwoordig erg veel geluisterd wordt naar de leerling (en zijn ouders) en amper naar de leraar is fnuikend voor de kwaliteit van het onderwijs (A. Brandsma).

Corry-Anke Brandsma, 8 september 2008

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Chapter 1

Introduction

Definition and epidemiology of COPD

Chronic Obstructive Pulmonary Disease (COPD) is a leading cause of death worldwide and its morbidity and mortality are still rising. According to the WHO, 80 million people suffer from moderate to severe COPD and three million people died of COPD in 2005. Furthermore, the WHO predicts that COPD will become the fourth leading cause of death worldwide by 2030 [1].

COPD is defined by the Global Initiative for Chronic Obstructive Lung Disease (GOLD) as "a preventable and treatable disease with some significant extrapulmonary effects that may contribute to the severity in individual patients. Its pulmonary component is characterized by airflow limitation that is not fully reversible. The airflow limitation is usually progressive and associated with an abnormal inflammatory response of the lungs to noxious particles or gases" [2].

COPD is mostly diagnosed in individuals above 40 years. The first signs of COPD are often chronic cough, increased sputum production, and dyspnea. The pulmonary manifestation of COPD is documented by a decrease in FEV_1 relative to the predicted normal FEV_1 and a decreased FEV_1/FVC ratio. GOLD has stratified the severity of COPD into stages I-IV [2]. Individuals with stage I have a mild airflow limitation and are often unaware of their decreased lung function. Individuals with stage IV have a severely limited lung function accompanied by symptoms like chronic dyspnea on exertion and in rest, chronic cough, and sputum production. In addition, respiratory insufficiency is present in a subset of COPD stage IV and exacerbations may be life threatening in these individuals.

Problem definition and aim

Smoking is widely accepted as the most important risk factor for the development of COPD in the Western world. Until now, the only effective treatment to diminish the accelerated lung function decline associated with COPD development and progression is smoking cessation. Nevertheless, the inflammatory response in the lungs persists after smoking cessation in COPD [3,4] and as yet it is not known whether and to what extent this contributes to ongoing disease progression. The mechanisms behind the smoke induced lung damage and the continuation of this inflammation after smoking cessation are largely unknown.

Next to smoking, other factors must be involved in COPD development as well, since also some non-smokers develop the disease and only a minority of smokers does. These factors include exposure to other toxic environments like air pollution, and genetic factors like α-1 antitrypsin deficiency and polymorphisms in genes encoding for proteases (matrix metalloproteases (MMPs)), antioxidants (gluthatione-S-transferases, heme oxygenase-1 (HO-1)) and inflammatory mediators (TNF-α, TGF-β) [2,5]. Whether these risk factors interact with cigarette smoke and to what extent they contribute to COPD development is currently unknown.

This lack of knowledge greatly hampers the development of new and effective treatments. To find better treatment targets for COPD patients and to understand why the chronic inflammatory response persists after stopping smoking, more

information is needed about the origin and nature of the chronic inflammatory response in COPD.
The aim of this thesis was to investigate both the early and late effects of cigarette smoking, together with the involvement of the air pollutant nitrogen dioxide (NO_2) and HO-1 in animal models for COPD. In addition, we investigated the role of the specific immune response, i.e. the involvement of B cells and regulatory T cells in the inflammatory response in COPD.
First an overview of the current knowledge on the pathogenesis of COPD will be given, followed by a rationale for studying the different aspects of the inflammatory response in COPD as assessed in this thesis.

Pathogenesis of COPD

COPD is a complex, heterogeneous disease consisting of chronic bronchitis, emphysema, and small airways disease. Chronic bronchitis and small airways disease are characterized by inflammation of the airways resulting in inflammatory cell infiltrates, chronic mucus production, and fibrosis that all contribute to airway obstruction. Emphysema is characterized by tissue destruction of the parenchyma leading to loss of alveolar attachments. This can lead to loss of elastic recoil and hence to airway obstruction, but also contributes to a decreased surface area for oxygenation, which can lead to a disturbed diffusion capacity. The loss of alveolar tissue is also associated with a local inflammatory response.
The pathogenesis of COPD encompasses three major injurious processes including an abnormal inflammatory response in the lungs and failure to resolve inflammation, a protease/antiprotease imbalance, and increased oxidative stress (oxidant/antioxidant imbalance), all contributing to persistent inflammation and ongoing lung matrix changes [6,7].
Proteases are produced by several inflammatory cells and can destroy matrix components of the lung, contributing to emphysema development. Important proteases linked with COPD are serine proteases e.g. neutrophil elastase, which is inhibited by α1- antitrypsin (α1-AT) and MMPs, in particular MMP-1, MMP-2, MMP-9 and MMP-12 [7-9].
Normally proteases are counteracted by antiproteases. Well known examples of antiproteases are α1-AT, secretory leukoprotease inhibitor (SLPI) and tissue inhibitors of metalloproteases (TIMPs). Inherited α1-AT deficiency leads to early onset emphysema and both α1-AT and SLPI can be inactivated by cigarette smoke and oxidative stress [9,10]. The increased TIMP-1 secretion from alveolar macrophages in response to inflammatory stimuli is blunted in cells derived from COPD patients, and there is an increased frequency of loss-of-function mutations of TIMP-2 in COPD patients [9,10].
Oxidative stress is a well known feature of COPD. The main external sources of oxidative stress are environmental exposures like cigarette smoke and air pollution. Additional endogenous producers of oxidative stress are inflammatory cells like activated neutrophils, macrophages and eosinophils. Oxidants are counteracted by antioxidants, of which the glutathione system is thought to be the most important in the airways. An additional antioxidant mechanism in the

lung is the increase in HO-1 activity after oxidative stress, resulting in high levels of the antioxidant bilirubin [9-11].
Since the inflammatory response is the central outcome parameter in all studies that will be described in this thesis, we will discuss the different inflammatory and resident cell types involved in the inflammatory response in COPD below, which will be followed by a more extensive introduction of the involvement of NO_2 exposure and HO-1 expression in COPD.

Epithelial cells

The epithelium is the first line of defense of the airways. Mucus production by goblet cells and secretion of defensins is important in the protection against microbial infections and inhaled particles. On the other hand, increased mucus production, from goblet cell and bronchial glands hyperplasia contributes to airway obstruction [12]. Additionally, epithelial cells are an important source of inflammatory mediators, particularly when activated by cigarette smoke exposure [13-16] and will contribute to the inflammatory environment in COPD. Next to the production of inflammatory mediators, bronchial epithelial cells have been shown to exhibit an increased TGF-β production in smokers [17], which can contribute to the development of airway wall fibrosis.

Neutrophils

Neutrophils are an important component of the innate immune system, mediating the acute inflammatory responses against bacterial infections. Neutrophils contain granules filled with enzymes and proteases which play a role in the phagocytosis and destruction of microbes. During phagocytosis reactive oxygen species (ROS) are formed as well. Increased numbers of neutrophils have been found in bronchoalveolar lavage (BAL), sputum, bronchial glands, and airway smooth muscle of patients with COPD [4,18-21]. In contrast, only a few reports showed increased numbers of neutrophils in the airways or the parenchyma of patients with COPD [22,23].
Large numbers of activated neutrophils in the lungs can result in increased levels of ROS and proteases. The increase in ROS can shift the balance between oxidants and antioxidants, and the increase in proteases can shift the protease-antiprotease balance, both in favour of lung destruction. Additionally, release of neutrophil elastase can stimulate macrophages to the release of even more ROS, causing epithelial damage and increased mucus production [24]. Neutrophil elastase itself can also directly stimulate mucus production by submucosal glands and goblet cells in the epithelium [25-27]. Both contribute to development of chronic bronchitis.

Macrophages

Like neutrophils, macrophages also are important regulators of the innate immune response against infections. Additionally, macrophages play a role in adaptive immunity by acting as antigen presenting cells producing cytokines and providing co-stimulation.
Increased numbers of macrophages have been found in the airways, parenchyma, BAL, and sputum of patients with COPD [4,9,22,23]. Interestingly, macrophage

numbers in lung tissue have been shown to positively correlate with COPD severity [22,23].
Macrophages are thought to play an important role in COPD development as they are activated by cigarette smoking to secrete several inflammatory substances i.e. ROS, proteases, cytokines, and chemokines. Increased cytokine and chemokine production can lead to increased leukocyte recruitment and in turn to the production of pro-inflammatory cytokines, which contributes to the chronic inflammatory reaction ultimately leading to tissue remodelling with destruction of parenchyma and airway wall fibrosis. Additionally, macrophages also can produce TGF-β which contributes to the development of airway wall fibrosis [9,28].

Eosinophils

The eosinophil is a granulocyte which is involved in allergic reactions and the protection against parasites. Increased numbers of eosinophils are often seen in asthma and allergic diseases. However, the relevance of eosinophils in the inflammatory response in COPD is uncertain. Nevertheless, increased eosinophil numbers have been reported in patients with COPD, and particularly so during exacerbations [29-31]. Furthermore, several eosinophilic products i.e. eosinophil cationic protein and eosinophil peroxidase can cause serious damage to the lungs, which may lead to inflammatory changes such as can be seen in COPD.

B lymphocytes

B lymphocytes are the key players of the adaptive immune system with respect to providing humoral immunity via the production of antibodies.
Bosken et al [32] were the first to detect B lymphocytes organized in follicle-like structures in the airway adventitia of smokers. More recently, Hogg and coworkers more extensively assessed the inflammation in the small airways of patients with COPD [33]. They described increased numbers of small airways containing B lymphocytes together with a marked increase of small airways with lymphoid follicles in GOLD stage III-IV compared to stage 0-II. Recently our group has demonstrated an increase of B lymphocytes in the mucosa of large airways of COPD patients compared to controls [34]. However, the role, and thus relevance, of these B lymphocytes and lymphoid follicles in COPD remains unclear.
We hypothesized that B lymphocytes contribute to the development of COPD by means of an antigen specific reaction. In order to test whether our hypothesis is tenable we studied the presence, organization, and clonality of these cells both in human lung tissue and in our mouse model of cigarette smoke-induced emphysema (chapter 4).

T lymphocytes

T lymphocytes are key players in the adaptive immune response with respect to regulation of cellular immunity. $CD4^+$ T lymphocytes control immune responses to extracellular antigens and function as helper T lymphocytes. $CD8^+$ T lymphocytes are involved in the response to intracellular microbes, i.e. viral, tumor, and certain microbial antigens, and function as cytotoxic T lymphocytes killing the cells that produce or harbor the foreign antigen.

Increased numbers of both $CD4^+$ and $CD8^+$ T lymphocytes are present in lung tissue of COPD patients, with a predominance of $CD8^+$ T lymphocytes [23,35-37]. In addition, the number of $CD8^+$ T lymphocytes is correlated with a decreased lung function [23,36]. One theory is that the increased recruitment of $CD8^+$ T lymphocytes may be a result of viral infections, which occur frequently in COPD patients [38]. Increased numbers of $CD8^+$ T lymphocytes may then contribute to tissue damage by their release of perforins and granzyme-B. $CD4^+$ T lymphocytes may contribute to the inflammatory response in COPD by the production of several pro-inflammatory cytokines or by acting as helper cells, priming $CD8^+$ T lymphocytes responses or maintaining immunological memory. By this immunological memory $CD4^+$ T lymphocytes could possibly be involved in the perpetuation of the inflammatory response after smoking cessation. Interestingly, Sullivan et al showed the presence of oligoclonal $CD4^+$ T lymphocytes in lung tissue of patients with severe emphysema. This indicates the presence of an antigen-specific T lymphocyte-response in the lungs of these patients and a possible role for the adaptive immune system in COPD [39].

Regulatory T cells

Regulatory T cells (Tregs) are a special subset of $CD4^+$ T lymphocytes, which are important in controlling immunological tolerance and preventing auto-immune reactions by inhibiting T-cell responses [40]. Additionally, Tregs can also directly suppress B cell responses without having to suppress the adjacent T cells [41], indicating that activated T cells are not the only target for Tregs. Dysfunction of Tregs can lead to auto-immune diseases, allergy, and chronic inflammatory diseases [40,42]. The currently best described subset of Tregs is that of the naturally occurring Tregs. Naturally occurring Tregs are generated in the thymus and express CD4, high levels of CD25 and Forkhead transcription factor 3 (Foxp3). The suppressive effects of Tregs are mediated by cell-cell contact and possibly also by HO-1 expression and membrane bound TGFβ [43-45]. Tregs have been the subject of investigations in allergy and asthma [46]. However, very little is known so far about their contribution to the chronic inflammatory response in COPD. Until now, only three studies have assessed the presence of Tregs in COPD and reported different findings. The first showed decreased $CD4^+CD25^+$ Tregs in lung tissue of emphysema patients compared to control subjects [47]. The second showed increased $CD4^+CD25^{bright}$ Tregs in BAL from COPD patients and healthy smokers compared to healthy never smokers [48], and the third study showed increased $CD4^+CD25^+$ Tregs in BAL of healthy smokers compared to COPD patients and never smokers [49]. Recently, we have found the presence of Foxp3 positive T cells as a component of lymphoid follicles in the lungs of patients with COPD (figure 1), which suggests a role for Tregs in controlling this local B cell response.

In chapter 5 the presence and phenotype of Tregs and B cells in peripheral blood of COPD patients and healthy volunteers will be discussed. We hypothesize that the B-cell reaction in COPD is antigen driven and contributes to progression of the disease, and that Tregs are involved in the suppression of this B cell mediated response.

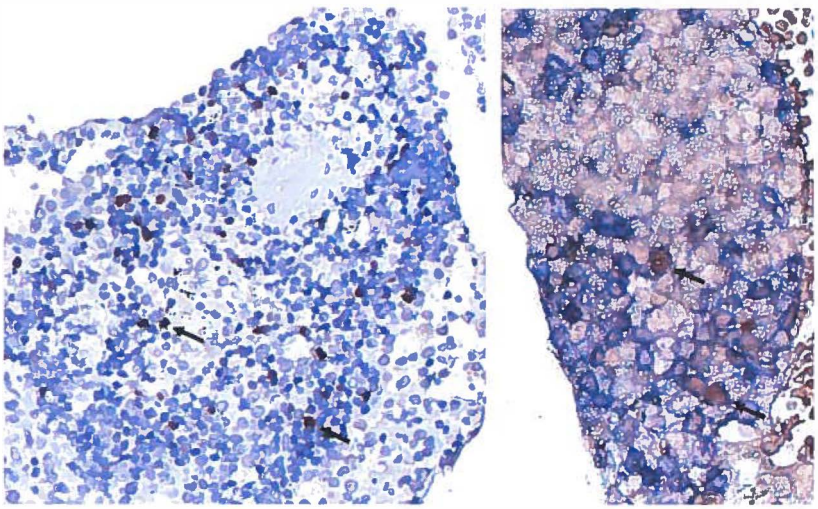

Figure 1. *Example of a single Foxp3(red) and double CD3(blue)/Foxp3(red) staining in a lymphoid follicle in a human COPD lung. Positive cells are indicated with an arrow.*

Nitrogen dioxide exposure and COPD

As already described, next to cigarette smoking, other environmental exposures such as nitrogen dioxide (NO_2) may contribute to COPD development. NO_2 is an important air pollutant, that has been associated with increased respiratory symptoms and decreased lung function in humans [50-53] and with emphysema development in animal models [54-58]. NO_2 is mainly formed by the combustion of fossil fuels. Motor vehicle and industry emissions are important outdoor sources of NO_2, whereas indoor cooking and heating with fossil fuels and cigarette smoke are important indoor sources [59-62]. It is difficult to disentangle the separate effects of exposure to different air pollutants and/or cigarette smoke in epidemiologic studies, because individuals are never exposed to a single pollutant. To what extent environmental NO_2 exposure contributes to the development of COPD and can add to the noxious effects of smoking is largely unknown. It is known however, that NO_2 exposure can form nitrate, nitrite, and nitrous acid in the epithelial lining of the lungs. When combined with cigarette smoke exposure, hydroxyl radicals are formed, which are extremely reactive and cause increased oxidative stress in the lungs [38].

To gain more insight into the contribution of NO_2 exposure to COPD development we studied the effects of NO_2, cigarette smoke, and their combined exposure on pulmonary inflammation and emphysema development in mice. We hypothesized that combined exposure to NO_2 and cigarette smoke would enhance pulmonary inflammation and emphysema development in mice. In chapter 2 the short term and in chapter 3 the long term effects of NO_2 and cigarette smoke exposure and their interaction are described.

Heme oxygenase-1 and COPD

An intriguing protein that may play a role in COPD development is the 'protective' enzyme heme oxygenase-1 (HO-1). HO-1 is the rate limiting enzyme involved in the breakdown of heme to equimolar amounts of bilirubin, free iron and carbon monoxide (CO). HO-1 is a stress response protein, which is rapidly upregulated after oxidative stress and has potent anti-oxidative, anti-inflammatory, anti-apoptotic and anti-proliferative effects [11,63,64]. The anti-inflammatory and cytoprotective effects of HO-1 are mediated by its products, of which in particular CO [65-67]. CO is a molecule with potent anti-inflammatory effects; in vitro and in vivo CO has been shown to inhibit LPS (lipopolysaccharide) induced TNF-α and IL-1β production, while increasing the IL-10 production [65]. The antioxidative effects are mainly mediated by the production of bilirubin, a well known antioxidant [8].

Notwithstanding this knowledge, the exact mechanisms behind the protective effects of HO-1 are still poorly understood.

Interestingly, in patients with COPD, a reduced HO-1 expression has been shown in macrophages in lung tissue and bronchoalveolar lavage (BAL) [68,69]. One of the possible explanations could be a genetic polymorphism in the HO-1 promoter gene, which is associated with a lower induction of HO-1 by reactive oxygen species (ROS) and is associated with emphysema development in a Japanese population [70].

We hypothesize that in COPD patients HO-1 is insufficiently upregulated leading to a higher vulnerability to the effects of cigarette smoke and oxidative stress. In chapter 6 we tested whether HO-1 modulation in our smoking mouse model could influence the development of cigarette smoke-induced emphysema and lung inflammation. We hypothesized that HO-1 induction attenuates cigarette smoke induced emphysema and inflammation and conversely HO-1 inhibition worsens the noxious effects of cigarette smoke.

Subsequently, we developed a new non-invasive method for HO-1 induction in mice, which is described in chapter 7.

Finally, all results described in this thesis are summarized and discussed in chapter 8, with a perspective to the future.

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Chapter 2

Short-term cigarette smoke exposure attenuates nitrogen dioxide induced pulmonary inflammation in mice

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Submitted

Abstract

Smoking is recognized as the most important cause for development of COPD. Other factors have to be involved as well, since some non-smokers and only a minority of smokers develops COPD. The air pollutant nitrogen dioxide (NO_2) is associated with respiratory symptoms and with emphysema in animal models. We hypothesized that NO_2 interacts with cigarette smoke to enhance pulmonary inflammation. This was studied in mice that were exposed to 40 puffs of cigarette smoke in 2.5 hours, 25 ppm NO_2 for 3 hours, or 25 ppm NO_2 for 3 hours followed by 40 puffs of cigarette smoke, or to air.

NO_2 exposure increased the levels of several pro-inflammatory cytokines and the numbers of eosinophils and neutrophils, and decreased levels of IL-10. Cigarette smoke exposure increased the numbers of macrophages and T cells, and the levels of IL-6, MCP-1, and GMCSF. Interestingly, the NO_2 induced cytokine production was abolished when NO_2 exposure was followed by cigarette smoke exposure. In an additional experiment, we showed that these effects can partly be mimicked by carbon monoxide, a component of cigarette smoke.

Introduction

Chronic obstructive pulmonary disease (COPD) is a leading cause of death worldwide and its morbidity and mortality are rising. Although the pathogenesis of the disease is still largely unknown, smoking is widely accepted as the most important cause for the development of the disease in the industrialized world. The majority of patients with COPD are current or ex smokers, yet only a minority of smokers eventually develops COPD. The factors causing these individuals to develop COPD are currently largely unknown.

Since not all COPD patients have smoked, cigarette smoke cannot be the sole contributing factor to disease development. Environmental factors may contribute as well and the air pollutant NO_2 is such a factor, known to be capable of inducing respiratory symptoms. NO_2 is mainly formed by the combustion of fossil fuels. Motor vehicle and industry emissions are important outdoor sources of NO_2, whereas cooking and heating with fossil fuels and cigarette smoke are important indoor sources [1-4]. Different epidemiological studies have shown associations between environmental pollution, NO_2 exposure, and level of lung function [5-7]. A recent study showed that increased environmental NO_2 exposure is associated with a reduced growth in forced expiratory volume in one second (FEV_1) in childhood [6] and Ponka et al described a positive association between levels of environmental NO_2 and numbers of emergency room admissions for COPD exacerbations [7].

In vitro and in vivo studies in mice and humans have shown that acute NO_2 exposure results in increased levels of the inflammatory cytokines TNF-α, IL-1β, IL-6, IL-8 and RANTES together with an increased expression of heme oxygenase-1 (HO-1) and inducible nitric oxide synthase (iNOS) [8-11]. Although chronic exposure to NO_2 has been shown to induce emphysema in some animal models [12-14] it is uncertain whether NO_2 exposure alone is sufficient to elicit full-blown emphysema in humans.

To provide more information on the first processes that initiate COPD and the role of environmental pollution, especially NO_2 exposure, in relation to cigarette smoke in this process, we studied the short-term effects of cigarette smoke and NO_2 exposure; either alone or in combination in mice. We hypothesized that NO_2 and cigarette smoke interact to enhance pulmonary inflammation.

Methods

Animals

Female A/J mice (aged 8-10 weeks) were obtained from Harlan (Horst, the Netherlands) and were held at the Central Animal Facility of the University of Groningen. The experiments were approved by the local Committee on Animal Experimentation and were performed under strict governmental and international guidelines on animal experimentation.

Study design

Four groups of mice (n=8 per group) were exposed to either 1) 40 puffs of cigarette smoke in 2.5 hours, 2) 25 ppm NO_2 for 3 hours, 3) 25 ppm NO_2 for 3 hours followed by 40 puffs of cigarette smoke in 2.5 hours, or 4) air. The mice were sacrificed seventeen hours after exposure. The left lung was removed, lung cells were isolated and used for flow cytometric analysis; the right lung was partly snap-frozen or Nakane-fixed and kept at -80°C until it was used for histology, cytokine and western blot analyses. Nakane fixation was used to obtain a better morphology for histology [15].

Smoke exposure

The smoke exposure system of the Tobacco and Health Research Institute of the University of Kentucky was used for the nose-only exposure of mice to mainstream smoke. The system was set up according to the instructions of the manufacturer for generation of mainstream smoke [16,17]. The smoking machine was checked for the delivery of total particulate matter (TPM) as described by Griffith et al. and calibrated before every smoking session to ensure accurate and standardized smoke delivery. The mice were placed in restrainers and subsequently exposed to 40 puffs of cigarette smoke from 4 2R1 Reference Cigarettes (University of Kentucky) during 2.5 hours. Control mice were sham-exposed in a separate animal exposure unit, which was placed under similar but smokeless circumstances. These mice were exposed to room air for the same 2.5 hours the smoke exposure took.

NO_2 exposure

The NO_2 exposure system was developed in co-operation with the National Institute of Public Health and the Environment (RIVM, Bilthoven, the Netherlands). The mice were placed in mesh cages within an airtight box, with a continuous flow (5.5 m3/hr) of NO_2. The desired NO_2 concentration of 25 ppm was generated by diluting liquid NO_2 with compressed air. The NO_2 concentration was measured using a nitrogen oxides analyzer (Eco Physics CLD 700AL, CleanAir, Marseille, France) and was kept at the desired range of 25 ppm +/- 10%. The mice were exposed to NO_2 for 3 hours. Control mice received the same treatment as NO_2 exposed mice; they were exposed to room air in similar mesh cages and in parallel with the NO_2 exposure. During the exposure mice freely moved and had access to water and standard rodent food ad libitum.

Cytokines

Frozen lung tissue was homogenized 5 or 10% w/v in a 50 mM Tris-HCl buffer, containing 150 mM NaCl, and 0.002% Tween-20 (pH 7.5) and centrifuged at 12000xg for 10 min to remove any insoluble material. Concentrations of murine IL-1β, IL-5, IL-6, IL-9, IL-10, IL-13, monocyte chemoattractant protein-1 (MCP-1), granulocyte-macrophage colony-stimulating factor (GM-CSF), RANTES and TNF-α in lung homogenate were measured with a multiplex ELISA system (Lincoplex Systems, St Charles, USA).

Flow cytometry

Single-cell leukocyte suspensions were obtained from lungs for flow cytometric analysis as described previously [18]. Cells were quantified using a Coulter Counter Z1 (Coulter, Hialeah, USA). Expressions of CD3, CD4, CD25, and B220 cell surface markers on isolated leukocytes were examined with four-color flow cytometry to determine frequencies of B cells and T-cell subsets. The leukocytes were stained with a combination of directly conjugated antibodies directed against these cell surface markers as described previously [18]. All antibodies were obtained from Pharmingen (San Diego, USA) and were conjugated with one of the following fluorochromes: FITC, phycoerythrin (PE), peridinin chlorphyll protein (PerCP), and allophycocyanin (APC). Frequencies of B cells and T-cell subsets were based on the label combinations: CD3-APC, CD4-PE, B220-PerCP, and CD25-FITC. Cell populations (4 x104 events) were analyzed using an Epics Elite flow cytometer (Coulter Epics, Hialeah, USA) and data analysis was performed using FlowJo software (ThreeStar, San Carlos, USA).

Histology

Eosinophil numbers were determined in 4 µm sections of frozen lung tissue by staining for cyanide resistant endogenous peroxidase activity with diaminobenzidine (Sigma-Aldrich, St. Louis, USA). Neutrophil and macrophage numbers were identified in 4 µm sections of Nakane-fixed frozen lung tissue with the rat monoclonal antibodies anti-GR1 (Pharmingen) and anti-Mac3 (Pharmingen) respectively. Eosinophils and macrophages were quantified by morphometric analysis and expressed as volume percentages [18]. Total numbers of neutrophils were scored and expressed as numbers of GR1 positive cells per microscopic field.

NO_2 + CO experiment

In order to unravel the dampening effects of cigarette smoke exposure on the NO_2 induced inflammation, we performed an additional, similar, experiment with carbon monoxide (CO) exposure. CO was chosen since it is known to have potent anti-inflammatory capacities [19], and is present in high concentrations in cigarette smoke [20].
Four groups of mice (n=8 per group) were exposed to either 1) 250ppm CO for 3 hours, 2) 25 ppm NO_2 for 3 hours, or 3) 25 ppm NO_2 for 3 hours followed by 250ppm CO for 3 hours, or 4) air. Mice were sacrificed after exposure and lung tissue was snap-frozen and kept at -80°C until it was used for cytokine analyses.

CO exposure

The mice were placed in a Plexiglas exposure chamber (5 liters) with a continuous flow of 250 ppm +/- 10% CO. Mice were exposed for 3 hours and could freely move during exposure. Control mice received the same treatment as CO exposed mice; they were exposed to room air in a similar exposure chamber and in parallel with the CO exposure.

Statistics

Multiple linear regression analysis was used to establish the statistical significances of differences in terms of NO_2, cigarette smoke and CO exposure for each parameter. This method disentangles the separate effects of NO_2, cigarette smoke, CO, and their interactions. A significant interaction signifies that the effect of the combination is different (larger or smaller) than the addition of the separate effects of the exposures. When the interaction was not significant, the regression analysis was performed again without this interaction term. The normal distribution of the residuals was tested with a Kolmogorov-Smirnov test and data were log -, $-x^2$, or 1/x-transformed when needed to normalize distributions. A value of $p<0.05$ was considered significant. Mann Whitney U tests were used for post-hoc analysis to test whether NO_2 exposure alone differed from NO_2 + CS or NO_2 + CO exposure.

Results

Effects of short-term NO_2 exposure on the presence of cytokines and inflammatory cells in the lung

Short-term NO_2 exposure significantly increased the levels of the pro-inflammatory mediators TNF-α, IL-1β, and IL-6, and of the cytokines involved in the recruitment, activation and differentiation of eosinophils like IL-5, IL-9, IL-13, RANTES, and MCP-1 in lung tissue (Figure 1 and Table 1). In addition, NO_2 exposure decreased the level of the anti-inflammatory cytokine IL-10. With respect to inflammatory cells, short-term NO_2 exposure increased the numbers of eosinophils and neutrophils in lung tissue (Figure 2A,B), but had no effect on the numbers of macrophages (Figure 2C), T cells, and B cells (Table 2).

Table 1: Cytokine protein levels in lung homogenate after NO_2 and cigarette smoke exposure.

Cytokines (pg/g lung tissue)	Values				P-values		
	Control	CS	NO_2	NO_2 + CS	CS effect	NO_2 effect	CS x NO_2 interaction
TNF-α	2.4+/-0.2	2.4+/-0.1	5.4+/-0.4	2.8+/-0.2 #	n.s.	< 0.01 ↑	< 0.01 ↓
IL-1β	8.0+/-1.0	8.0+/-0.6	16.7+/-1.3	9.1+/-1.1 #	n.s.	< 0.01 ↑	< 0.01 ↓
IL-9 *	30.6+/-11.4	25.8+/-1.0	90.1+/-11.3	25.8+/-2.7 #	n.s.	< 0.01 ↑	< 0.01 ↓
IL-13	9.3+/-0.7	8.8+/-0.3	12.0+/-0.5	8.4+/-0.8 #	n.s.	< 0.01 ↑	< 0.01 ↓
IL-10	96.6+/-14.1	104.0+/-7.6	60.1+/-8.7	130.0+/-11.1 #	n.s.	< 0.05 ↓	< 0.01 ↑
GM-CSF *	12.8+/-3	37.9+/-15.3	10.4+/-0.4	21.4+/-3.4	< 0.01 ↑	< 0.05 ↓	n.s.

*Values expressed as mean +/- standard error of the mean (SEM). * indicates that the data were not normally distributed and geometric mean +/- SEM is depicted. P-value <0.05 is considered significant. The arrows indicate the direction of the effect. A significant interaction signifies that the value in the group that received both NO_2 and cigarette smoke is either significantly greater (arrow pointing up) or significantly smaller (arrow pointing down) than the added effects of NO_2 and cigarette smoke. # indicates a significant difference between NO_2 exposure and NO_2 + CS exposure. CS = cigarette smoke exposure. n.s. = not significant.*

Effects of short-term cigarette smoke exposure on the presence of inflammatory cells and cytokines in the lung

Short-term cigarette smoke exposure increased the numbers of macrophages (Figure 2C), $CD4^+$ T cells, $CD4^+CD25^+$ T cells, and $CD8^+$ T cells in lung tissue, with a trend for an increase in B cells (Table 2). Smoking had no effect on the numbers of neutrophils and eosinophils in lung tissue (Figure 2A,B). Additionally, smoking significantly increased the levels of IL-6, MCP-1 and GM-CSF in lung tissue (Figure 1 and Table 1).

Short-term cigarette smoke exposure abolishes NO_2 induced cytokine production

There were significant negative interactions between the effect of NO_2 exposure and the effect of cigarette smoke exposure for the levels of TNF-α, IL-1β, IL-5, IL-6, IL-9, IL-13, RANTES, and MCP-1 in lung tissue (Table 1 and Figure 1). These interactions are the result of increased cytokine levels after NO_2 exposure alone and the lack of such an effect in animals exposed to the combination of NO_2 and cigarette smoke (NO_2 vs. NO_2 + CS, $p< 0.05$). Additionally, there was a significant positive interaction between the effect of NO_2 exposure and the effect of cigarette smoke exposure for the level of IL-10, which is caused by a decrease in IL-10 after NO_2 exposure alone and an increase in animals exposed to the combination of NO_2 and cigarette smoke (Table 1) (NO_2 vs. NO_2 + CS, $p< 0.05$). These interactions signify that the effect of NO_2 exposure on cytokine production was different when it was followed by cigarette smoke exposure. Smoking had no significant dampening effect on the NO_2 induced increase in numbers of eosinophils and neutrophils in the lung (Figures 2A,B).

Table 2: Lymphocytes in lung homogenate after NO_2 and cigarette smoke exposure.

Lymphocytes (cell numbers per mg lung tissue)	Values				P-values		
	Control	CS	NO_2	NO_2 + CS	CS effect	NO_2 effect	CS x NO_2 interaction
$CD4^+$ T cells	518+/-71.5	733+/-96.5	616+/-98.7	968+/-192.9	< 0.05 ↑	n.s.	n.s.
$CD4^+CD25^+$ T cells	25.5+/-5.0	42.3+/-7.3	29.2+/-4.7	57.6+/-10.1	< 0.01 ↑	n.s.	n.s.
$CD8^+$ T cells	239+/-33.1	401+/-50.3	334+/-64.4	480+/-99.2	< 0.01 ↑	n.s.	n.s.
B cells	872+/-139	1373+/-216	1275+/-247	1792+/-391	p=0.06 ↑	n.s.	n.s.

Values expressed as mean +/- SEM. P-value <0.05 is considered significant. The arrows indicate the direction of the effect. CS = cigarette smoke exposure. n.s. = not significant.

Effects of short-term CO exposure on inflammatory cytokines in lung tissue

To investigate whether the inhibitory effects of cigarette smoke exposure could be explained by the presence of CO in cigarette smoke, the model was repeated with CO exposure instead of cigarette smoke exposure. CO exposure inhibited the NO_2 induced increase in the level of MCP-1 in lung tissue (NO_2 vs. NO_2 + CO, $p< 0.05$), but had no effects on NO_2 induced increases in the levels of IL-6 and IL-5 (Figure 3). Additionally CO exposure decreased the levels of MCP-1 and RANTES, and there was a trend for IL-5 (Figure 3).

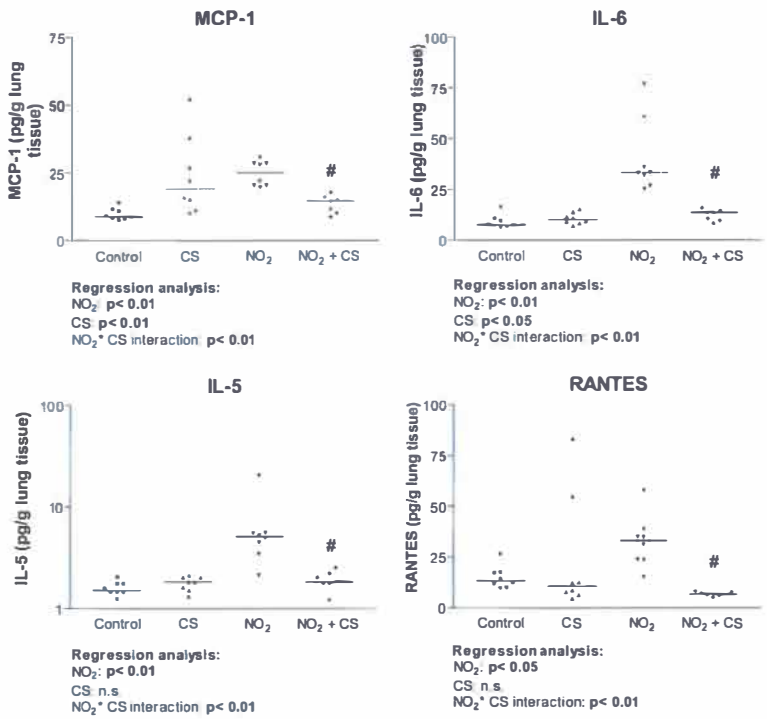

Figure 1. *Cytokines in lung homogenate after NO_2 and cigarette smoke exposure.* *MCP-1, IL-6, IL-5 and RANTES levels expressed as pg/g lung homogenate after NO_2 and cigarette smoke exposure. # indicates a significant difference between NO_2 exposure and NO_2 + CS exposure. Results of the regression analysis are depicted beneath the figures. CS = cigarette smoke exposure. n.s. = not significant*

Discussion

In this study we showed that short-term NO_2 exposure elicits increased levels of pro-inflammatory cytokines in the lung, while short-term cigarette smoke exposure elicits increased numbers of inflammatory cells in the lung. Intriguingly, the NO_2 induced cytokine production was completely abolished when NO_2 exposure was followed by short-term cigarette smoke exposure. We demonstrated that this can partly be explained by CO, which is present in cigarette smoke.

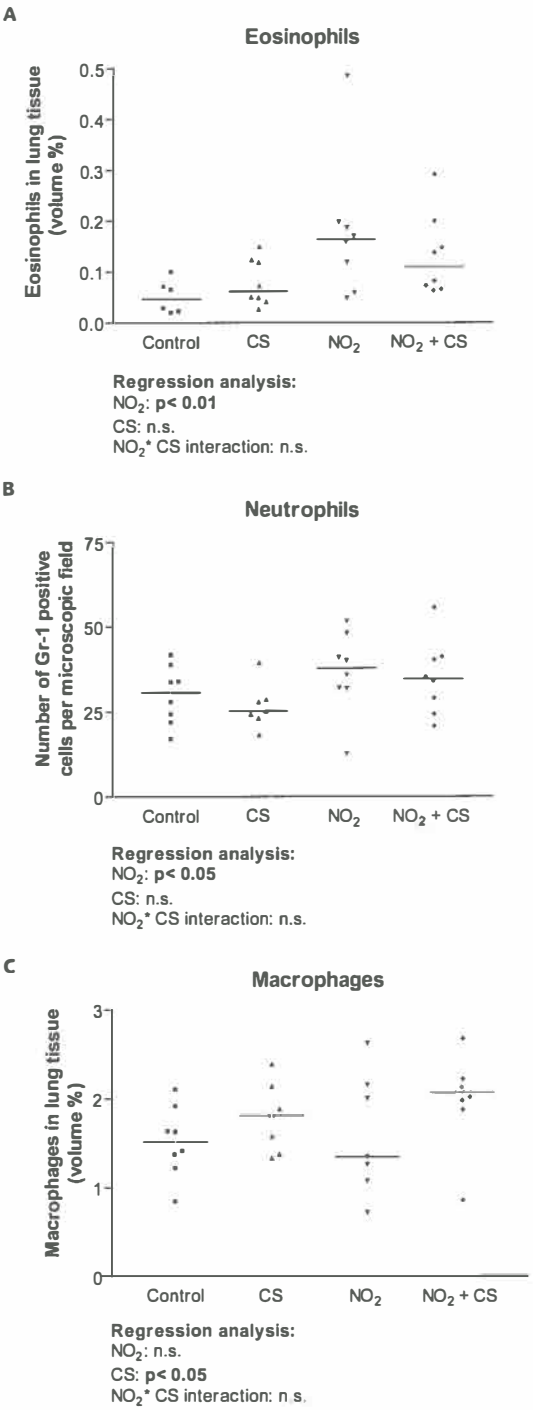

Figure 2. ***Inflammatory cells in lung tissue after NO_2 and cigarette smoke exposure.***
A) Eosinophils expressed as volume percentages, B) Neutrophils expressed as numbers of Gr-1 positive cells per microscopic field, and C) Macrophages expressed as volume percentages in lung tissue after cigarette smoke and NO_2 exposure. Results of the regression analysis are depicted beneath the figures. CS = cigarette smoke exposure. n.s. = not significant

There was a negative interaction between short-term NO_2 and cigarette smoke exposure with respect to levels of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) and cytokines involved in eosinophil recruitment, activation and differentiation (IL-5, IL-9, IL-13, RANTES, and MCP-1). These negative interactions are in contrast to our original hypothesis, i.e. NO_2 and cigarette smoke interact positively to enhance pulmonary inflammation. However, these unexpected results are in line with more recently published data in which we and others have shown dampening effects of smoking on ovalbumin (OVA)-induced airway inflammation in mice [21,22].

A possible explanation for the 'down regulatory' effects of cigarette smoke on the NO_2-induced inflammatory response is the presence of anti-inflammatory molecules in cigarette smoke, e.g. carbon monoxide (CO), nitric oxide (NO) [23] and nicotine [24-26]. CO is present in high concentrations in cigarette smoke and can also be produced locally in the lung by heme oxygenases (HO).

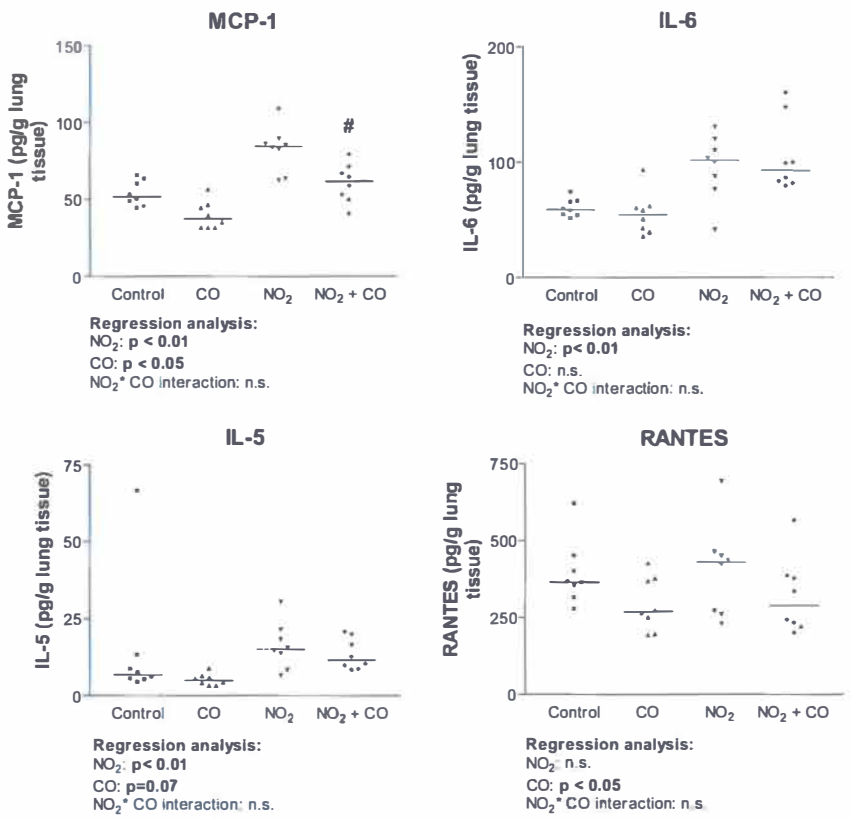

Figure 3: *Cytokines in lung homogenate after NO_2 and CO exposure.*
MCP-1, IL-6, IL-5 and RANTES levels expressed as pg/g lung homogenate after NO_2 and CO exposure. # indicates a significant difference between NO_2 exposure and NO_2 + CO exposure. Results of the regression analysis are depicted beneath the figures. n.s. = not significant

CO is a molecule with potent anti-inflammatory effects. It has been shown to inhibit lipopolysaccharide (LPS) induced TNF-α and IL-1β production in vitro and in vivo, while it increased IL-10 production [19]. This is compatible with the effects on cytokine production we found after exposing mice to NO_2 followed by cigarette smoke, i.e. a decrease in TNF-α and IL-1β levels together with an increase in IL-10.

To investigate whether CO was involved in the inhibitory effects of cigarette smoke in our model, the experiment was repeated with CO instead of cigarette smoke exposure. In this experiment we focused on the effects of CO exposure on cytokine levels. CO exposure inhibited the NO_2 induced increase in the levels of MCP-1, which is in accordance with the results found after smoke exposure. However, CO exposure had no effect on NO_2 induced increases in the levels of IL-5 and IL-6. Therefore, the 'down regulatory' effects of cigarette smoke can only partly be explained by the presence of CO in cigarette smoke. Nevertheless, CO exposure decreased the levels of MCP-1, RANTES, and IL-5, showing the anti-inflammatory capacity of CO.

In this model, short-term NO_2 exposure increased the levels of the pro-inflammatory cytokines TNF-α, IL-1β and IL-6. These results correspond with human in vitro and in vivo studies showing that acute NO_2 exposure results in increased levels of TNF-α, IL-1β, IL-6 and IL-8 [9-11]. After short-term NO_2 exposure, we also found increased levels of the cytokines IL-5, IL-9, IL-13 and chemokines RANTES and MCP-1 together with increased numbers of eosinophils in the lung. These results are similar as found in other studies assessing short-term NO_2 exposure. RANTES was shown to be increased in bronchial epithelial cells of asthmatic subjects after short-term NO_2 exposure [27] and IL-5 and IL-13 were shown to be increased in the epithelium of healthy human airways after repeated NO_2 exposure [28]. Additionally, short-term NO_2 exposure increased mRNA expression of RANTES, eotaxin, MIP-1α, MIP-2, interferon-γ-inducible protein-10 (IP-10) and MCP-1 in mice [8] and overexpression of IL-9 together with IL-9 induced chemokine expression (i.e. eotaxin, MIP-1α, MCP-1, -3 and -5) was described to be associated with airway eosinophilia [29-31].

In this model, the numbers of macrophages, $CD4^+$ T cells, $CD4^+CD25^+$ T cells and $CD8^+$T cells increased in the lung after short-term cigarette smoke exposure. This increased cellular influx after cigarette smoke exposure is compatible with other studies assessing the effects of short-term smoke exposure in mice. Increased numbers of macrophages have been shown in bronchoalveolar lavage (BAL) after one day exposure [32,33]. Effects on numbers of lymphocytes were not reported in these one day studies. However, increased numbers of lymphocytes in BAL and increased numbers of activated CD4 and CD8 T cells in lung tissue were shown after 1-or 2-week smoke exposure [34,35]. In contrast to other in vivo models of acute cigarette smoke exposure [32,34,36], we did not find increased neutrophil numbers after cigarette smoke exposure. This difference in effect on neutrophils can be explained by different time points of assessment i.e. 17 hours after exposure in our study versus 24 hours in the others. Our study also differed with respect to measurement of inflammatory cells in different lung compartments (lung tissue versus BAL fluid), and with respect to the mouse strain (AJ vs. C57BL/6). The latter is important as it has now been shown that susceptibility to cigarette smoke

induced lung inflammation and emphysema development is strain dependent in mice [37].
In conclusion, we showed strong, but different, inflammatory responses after short-term NO_2 and cigarette smoke exposure in mice. After long term exposure, both might contribute to the development of COPD. In contrast to our original expectation, most of the inflammatory effects of NO_2 exposure were abolished when NO_2 exposure was followed by cigarette smoke exposure. Since COPD is a chronic disease, clinically manifest after 20 years of smoking, we suggest that after chronic exposure, as opposed to our one-day exposure, the initial dampening effects of cigarette smoke will be gone and the combination of cigarette smoke and NO_2 will cause increased airway inflammation and enhanced development of COPD.

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Chapter 3

Nitrogen dioxide exposure attenuates cigarette smoke-induced cytokine production in mice

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Abstract

Cigarette smoke is the most important cause for the development of chronic obstructive pulmonary disease (COPD). Since only a minority of smokers and some nonsmokers develop COPD, other factors must be involved as well. NO_2 is an important air pollutant associated with respiratory symptoms in humans and emphysema development in animal models. We hypothesized that combined exposure to NO_2 and cigarette smoke will enhance pulmonary inflammation and emphysema development. Mice were exposed to 20 ppm NO_2 for 17 h/day, to 24 puffs of cigarette smoke 2 times per day, to their combination, or to control air for 5 days/wk during 4 wk. Following the last NO_2 exposure and within 24 h after the last smoke exposure the mice were sacrificed. Lungs were removed and analyzed for several inflammatory parameters and emphysema. Cigarette smoke exposure increased eosinophil numbers and levels of tumor necrosis factor (TNF)-α, KC, monocyte chemoattractant protein (MCP)-1, and interleukin (IL)-6. NO_2 exposure increased goblet cells, eosinophils, and the levels of IL-6, while it decreased the levels of IL-10. Four weeks of NO_2, cigarette smoke, or their combination was not sufficient to induce significant emphysema, nor did it lead to increased numbers of lymphocytes, neutrophils, or macrophages in lung tissue. Instead, NO_2 exposure attenuated the smoke-induced increases in levels of TNF-α, KC, and MCP-1. These dampening effects of NO_2 may be due to modulating effects of NO_2 on cytokine production by macrophages and epithelial cells, which have been reported earlier. The next step is to translate these findings of combined, controlled exposure in animals to the human situation.

Introduction

Chronic obstructive pulmonary disease (COPD) is a major global health problem with an increasing morbidity and mortality [1]. In western countries smoking is widely accepted as the most important cause for the development of the disease, still only a minority of the smoking population eventually develops COPD and the factors causing these individuals to develop COPD are currently unknown. Besides smoking, other factors that may contribute to COPD development are genetic factors, such as α1 anti-trypsin deficiency, and environmental factors like air pollution.

Animal models for COPD are crucial to explore the underlying mechanisms of the inflammatory response in COPD. Several models have been tested in the past, using different species and different experimental approaches [2,3]. Since cigarette smoking is considered the most important risk factor for COPD development, in particular smoke inhalation models have been used to study COPD pathogenesis [4-10]. Whole body and nose only smoke exposure models have been described and a minimum exposure period of 4 to 6 months was shown to be necessary to induce significant emphysema development, which is quite a long period for animal models. In addition to cigarette smoke, other inhalation atmospheres have been employed in the development of inhalation models for COPD as well, among them nitrogen dioxide (NO_2). NO_2 is an important environmental air pollutant, and is associated with increased respiratory symptoms and decreased lung function in humans [11-14] and with emphysema development in animal models [15-19].

In epidemiologic studies it is difficult to disentangle the separate effects of exposure to different air pollutants and/or cigarette smoke, because people are never exposed to a single pollutant. To what extent environmental NO_2 exposure contributes to the development of COPD and can add to the noxious effects of smoking is therefore largely unknown. Nevertheless, NO_2 exposure is known to form nitrate, nitrite, and nitrous acid in the epithelial lining of the lungs. When combined with smoke exposure, hydroxyl radicals are formed, which are extremely reactive and cause oxidative stress in the lungs [20]. To gain better insight into the contribution of NO_2 exposure to COPD development, we studied the effects of combined NO_2 and cigarette smoke exposure on pulmonary inflammation and emphysema development in mice. For this purpose mice were exposed for four weeks to NO_2 and cigarette smoke separately or to the combination of both agents. We hypothesized that combined exposure to NO_2 and cigarette smoke enhances pulmonary inflammation and emphysema development.

Methods

Animals

Female A/J Ola HSD mice (aged 8-10 weeks), which are known to be sensitive to the effects of cigarette smoke [8,10], were obtained from Harlan (Horst, the Netherlands) and were held at the Central Animal Facility of the Groningen University. The experiments were approved by the local Committee on Animal

Experimentation and were performed under strict governmental and international guidelines on animal experimentation.

Study design

Four groups of mice (n=12 per group) were exposed to either 1) air, 2) 24 puffs of cigarette smoke twice daily, 5 days a week, 3) 20ppm NO_2 continuously from 3pm until 8am, 5 days a week, or 4) 20ppm NO_2 continuously from 3pm until 8pm in combination with 24 puffs of cigarette smoke twice daily, 5 days a week.
Following the last NO_2 exposure and within 24 hours after the last smoke exposure the mice were sacrificed. The trachea was cannulated, the right lung was ligated, and lung lobes were either snap-frozen and stored at -80°C (n=8) or used directly for flow cytometry analysis (n=8). The left lung was inflated, and fixed for 24h with formalin with a constant pressure of 25 cm H_2O (n=8).

NO_2 exposure

The NO_2 exposure system was developed in collaboration with the National Institute of Public Health and the Environment (RIVM, Bilthoven, the Netherlands). The mice were placed in mesh cages within an airtight box, with a continuous flow (5.5 m3/hr) of NO_2. The desired NO_2 concentration of 20ppm was generated by diluting liquid NO_2 with compressed air. The NO_2 concentration was measured using a nitrogen oxides analyzer (Eco Physics CLD 700AL, CleanAir, Marseille, France) and was kept at the desired range +/- 10%. The mice were exposed to NO_2 continuously from 3pm until 8am, 5 days a week, for a period of 4 weeks. Control mice received the same treatment as NO_2 exposed mice; they were exposed to room air in similar mesh cages and in parallel with the NO_2 exposure. During the exposure mice could freely move and had access to water and standard rodent food *ad libitum*.

Smoke exposure

The smoke exposure system of the Tobacco and Health Research Institute of the University of Kentucky was used for the nose only exposition of mice to mainstream smoke. The system was set up according to the instructions of the manufacturer for generation of mainstream smoke [21,22]. The standard puffing regimen of 35ml puffs of 2.1 sec. taken once a minute was used and the cigarette smoke was diluted 1:1 with fresh air before exposure. The smoking machine was checked for the delivery of total particulate matter (TPM) as described by Griffith et al. and calibrated before every smoking session to ensure accurate and standardized smoke delivery. Average TPM values ranged between 38 and 40 mg per cigarette. Actual smoke exposure was assessed by measuring carboxyhemoglobin levels, which ranged from 15-35 % immediately after smoke exposure. The mice were placed in restrainers and subsequently exposed to 24 puffs of cigarette smoke from two 2R1 Reference Cigarettes (University of Kentucky, unfiltered, containing 32.9 mg tar and 2.19 mg nicotine per cigarette) two times per day, 5 days a week, for a period of 4 weeks. Control mice were sham-exposed in a separate animal exposure unit, which was placed under similar but smokeless circumstances. These mice were exposed to room air for the same 24 minutes the smoke exposure took.

Morphometrical evaluation of emphysema

Three-micron paraffin sections were cut and stained with haematoxylin and eosin. Approximately 25-30 photo microscopic images per tissue section were prepared at 2.5x20 magnification using a standardized sequence of image capturing. Alveolar airspace enlargement was assessed by mean linear intercept (Lmi) by two independent individuals in a blinded manner [23]. Images with large vessels, conducting airways or pleura occupying 25% or more of the total image were not used.

Flow cytometric analysis

Single-cell leukocyte suspensions were obtained from lungs for flow cytometric analysis as described previously [24]. Leukocytes were stained with a combination of directly conjugated antibodies directed against the following cell surface markers CD4-PerCP, CD25-FITC, B220-APC, and Gr1-APC to determine the frequencies of different T-cell subsets, B cells, and neutrophils. All antibodies were obtained from Pharmingen (San Diego, USA). Cell populations (4 x104 events) were analyzed using an Epics Elite flow cytometer (Coulter Epics, Hialeah, FL) and data analysis was performed using FlowJo software (ThreeStar, San Carlos, CA).

Histology

Macrophage numbers were identified in 3 µm sections of paraffin embedded lung tissue with the rat monoclonal antibody anti-Mac3 (Pharmingen). Eosinophil numbers were determined in 4 µm sections of frozen lung tissue by staining for cyanide resistant endogenous peroxidase activity with diaminobenzidine (Sigma-Aldrich, St. Louis, USA). Eosinophils and macrophages were quantified by morphometric analysis and expressed as volume percentages [24]. Goblet cells were determined in 3 µm sections of paraffin embedded lung tissue by a Periodic Acid Schiff (PAS) staining. The total number of PAS positive cells was counted and expressed per mm airway, as determined by morphometric analysis.

Cytokines

Frozen lung tissue was homogenized in 50 mM Tris-HCl buffer, containing 150 mM NaCl, and 0.002% Tween-20 (pH 7.5) and centrifuged at 12000xg for 10 min to remove any insoluble material. Concentrations of TNF-α, IL-6, KC (mouse IL-8), IL-10, and MCP-1 (monocyte chemoattractant protein-1) in the supernatants were measured with a multiplex ELISA system (Lincoplex Systems, St Charles, MO, USA).

Statistics

Multiple linear regression analysis was used to establish the statistical significances of differences in terms of NO_2 and cigarette smoke exposure for each parameter. This method disentangles the separate effects of cigarette smoke and NO_2 and their interaction. A significant interaction signifies that the effect of the combination is different (larger or smaller) than the addition of the separate effects of the exposures. When the interaction was not significant, the regression analysis was performed again without this interaction term. The normal distribution of the residuals was tested with a Kolmogorov-Smirnov test

and data were log -or 1/x-transformed when needed to normalize distributions. A value of $p<0.05$ was considered significant.

Results

Effects of NO_2 and/or cigarette smoke exposure on inflammatory cells in the lung

Both cigarette smoke ($p< 0.05$) and NO_2 ($p< 0.01$) exposure increased the numbers of eosinophils in lung tissue (Figure 1). Additionally, there was a significant negative interaction between NO_2 and smoking ($p< 0.01$) caused by a decreased number of eosinophils in the mice that were exposed to both NO_2 and cigarette smoke, when compared to NO_2 exposure alone.

NO_2 exposure increased the numbers of goblet cells, while there was no effect of smoking and no interaction between smoking and NO_2 exposure (Figure 2, $p< 0.05$).

Both NO_2 and/or cigarette smoke exposure had no effects on the numbers of neutrophils, macrophages, and different lymphocyte subsets in lung tissue (data not shown).

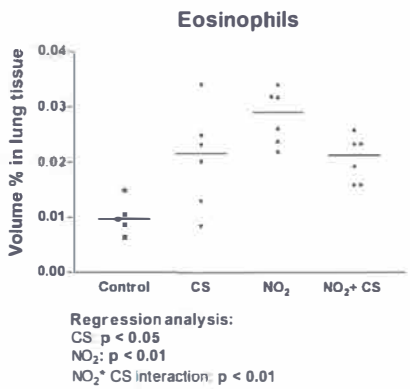


Figure 1. *Eosinophils expressed as volume percentages after subchronic NO_2 and/or cigarette smoke exposure. Results of the regression analysis are depicted beneath the figures. CS: cigarette smoke.*

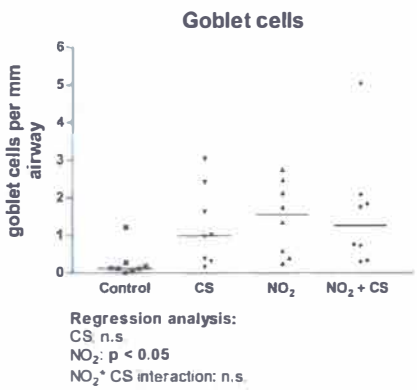
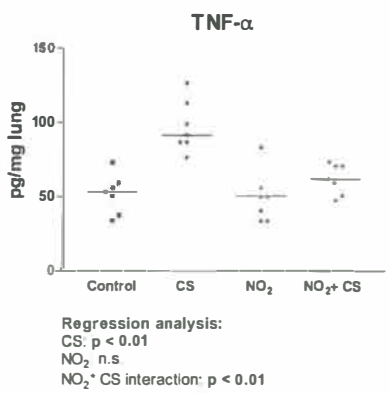


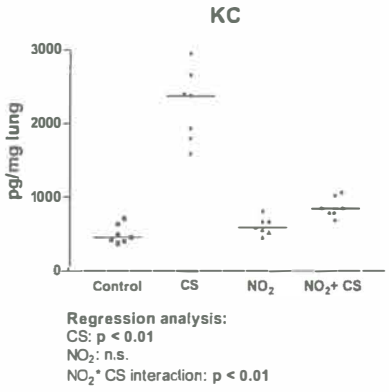
Figure 2. *Goblet cell numbers per mm airway after subchronic NO_2 and/or cigarette smoke exposure. Results of the regression analysis are depicted beneath the figures. CS: cigarette smoke. n.s. = not significant.*

Effects of NO_2 and/or cigarette smoke exposure on cytokine levels in the lung

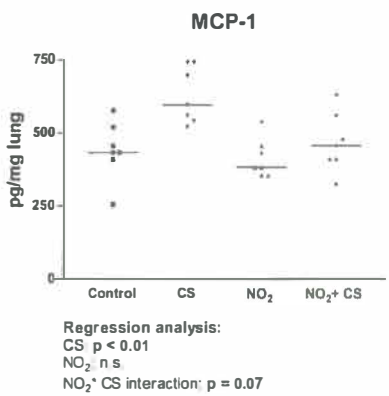
Cigarette smoke exposure significantly increased the levels of TNF-α, KC, and MCP-1 in lung homogenate ($p< 0.01$), while NO_2 exposure had no effects on these cytokines (Figure 3). Additionally, NO_2 exposure significantly decreased



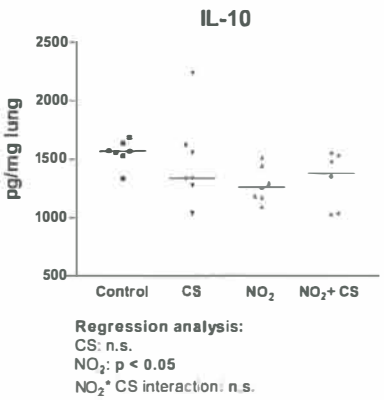
Regression analysis:
CS: $p < 0.01$
NO_2: n.s.
NO_2* CS interaction: $p < 0.01$



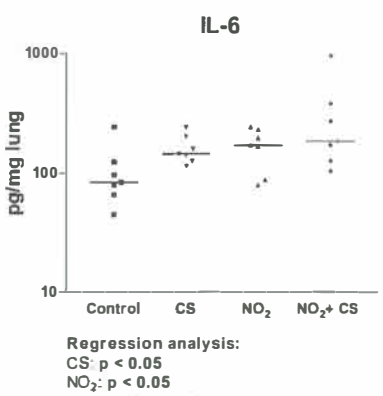
Regression analysis:
CS: $p < 0.01$
NO_2: n.s.
NO_2* CS interaction: $p < 0.01$



Regression analysis:
CS: $p < 0.01$
NO_2: n.s.
NO_2* CS interaction: $p = 0.07$



Regression analysis:
CS: n.s.
NO_2: $p < 0.05$
NO_2* CS interaction: n.s.



Regression analysis:
CS: $p < 0.05$
NO_2: $p < 0.05$
NO_2* CS interaction: n.s.

Figure 3. *TNF-α, KC, MCP-1, IL-10, and IL-6 levels expressed as pg/g lung homogenate after subchronic NO_2 and/or cigarette smoke exposure. Results of the regression analysis are depicted beneath the figures. CS: cigarette smoke. n.s. = not significant.*

the levels of the anti-inflammatory cytokine IL-10 ($p< 0.05$) and both NO_2 and cigarette smoke exposure increased the levels of IL-6 ($p< 0.05$).
There was a significant negative interaction between NO_2 exposure and smoking for the levels of TNF-α and KC ($p< 0.01$) and a trend for MCP-1 ($p=0.07$), which was caused by the increased cytokine levels after cigarette smoke exposure and the lack of such an effect in the mice that were exposed to both NO_2 and cigarette smoke.

Effects of NO_2 and/or cigarette smoke exposure on emphysema development

Four weeks of exposure to NO_2, cigarette smoke or their combination was not sufficient to elicit significant emphysema in this mouse strain (Figure 4).

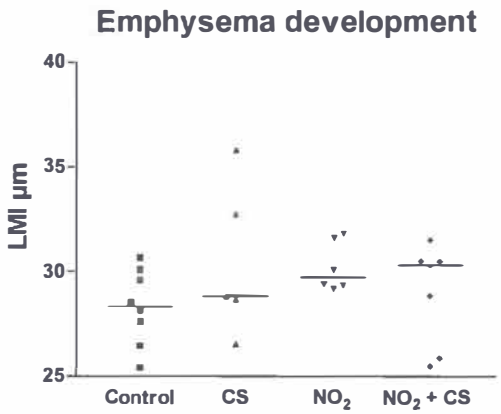


Figure 4. *Mean linear intercept (LMI) after subchronic NO_2 and/or cigarette smoke exposure. There were no significant effects of NO_2 or cigarette smoke exposure. CS: cigarette smoke.*

Discussion

In this study we hypothesized that the combination of NO_2 and cigarette smoke exposure would lead to an enhanced inflammatory response and more pronounced emphysema in mice. Contradictory to our hypothesis, NO_2 exposure dampened the cigarette smoke induced increases in the inflammatory cytokines TNF-α and KC.
These dampening effects can be due to immune modulatory effects of NO_2 which have been described earlier [11,25,26]. NO_2 exposure can impair the function of macrophages and epithelial cells, leading to increased susceptibility to infections and development of alternatively activated macrophages. Kienast et al. showed that NO_2 exposure decreased LPS induced pro inflammatory cytokine production

by alveolar macrophages in vitro, while baseline cytokine levels produced by non-stimulated macrophages were not affected by NO_2 exposure [25]. These results correspond well with our in vivo results, showing no effects of NO_2 exposure alone, but a dampening effect of NO_2 in combination with cigarette smoke. Probably, macrophage and epithelial cell function is impaired after subchronic NO_2 exposure leading to decreased pro inflammatory cytokine production. The latter then only becomes apparent after an inflammatory stimulus, like cigarette smoke exposure in this study. By decreasing the immune response to infections, NO_2 exposure may also lead to enhanced susceptibility to exacerbations in patients with COPD, since the majority of exacerbations is associated with viral or bacterial infections [27]. This is in accordance with data showing associations between hospital admissions for COPD exacerbations and levels of air pollutants like NO_2 [28].

Four weeks of cigarette smoke exposure resulted in increased levels of TNF-α, KC, IL-6, and MCP-1 together with increased numbers of eosinophils. This increase in eosinophils is interesting since increased presence of eosinophils has been reported in patients with COPD, and particularly so during exacerbations [29-31]. The importance of eosinophils in the inflammatory response in stable COPD and during exacerbations is controversial and the mechanisms and consequences remain to be elucidated [29]. Nevertheless, several eosinophilic products can cause serious damage to the lungs, which may lead to inflammatory changes as can be seen in COPD.

There was no effect of cigarette smoke exposure on numbers of neutrophils, macrophages, and lymphocytes, nor did it result in emphysema development. The latter is not surprising since there 4 to 6 months of smoke exposure was shown to be necessary to establish significant emphysema in other smoking mouse models [4-9]. However, the lack of an effect of smoking on neutrophils, macrophages, and lymphocytes is remarkable, since both cell types have shown to be increased after smoke exposure in other studies [4,32]. The lack of an effect in this study could be explained by the fact that inflammatory cells were measured in lung tissue versus BAL fluid in the other studies, by the difference in smoke exposure (whole body versus nose only), and by the fact that different mouse strains were being used. The latter is important as it has been shown that susceptibility to cigarette smoke induced lung inflammation and emphysema development is strain dependent in mice [8].

Four weeks of NO_2 exposure resulted in increased numbers of goblet cells and eosinophils, together with a small increase in the levels of IL-6 and decreased levels of IL-10.

Goblet cells are mucous producing cells and goblet cell hyperplasia and increased mucous production are an important characteristic of COPD [33]. Although, this study did not show emphysema development and increases in neutrophils and macrophages after subchronic NO_2 and cigarette smoke exposure, the increased number of goblet cells suggests a first step to a chronic bronchitis model.

The lack of increased levels of inflammatory cytokines after NO_2 exposure is in contrast to our model with acute NO_2 exposure (one day exposure to 25ppm for 3 hours)[34], in which several pro-inflammatory cytokines like TNF-α, KC, IL-6 and IL-1β were increased after NO_2 exposure. Other in vitro and in vivo studies have

shown increased levels of inflammatory cytokines after acute NO_2 exposure as well [35-39], whereas Garn et al showed decreased levels of TNF-α and IL-10 after subchronic NO_2 exposure in rats [26]. These findings indicate that there might be a difference in cytokine responsiveness between acute and subchronic NO_2 exposure. Additionally, after isolation of the macrophages Garn et al showed that these cells produced less pro-inflammatory cytokines in response to LPS in vitro, classifying these macrophages as alternatively activated.

In contrast to our expectations, subchronic NO_2 exposure alone did not result in significant emphysema development in this model. In several rat models [15,16,18] and recently also in a mouse model [19], significant emphysema development was shown after 4 weeks of NO_2 exposure. In these models, emphysema development was often accompanied by increased numbers of neutrophils and macrophages, while in our study only increases in goblet cells were found and no effects on neutrophils and macrophages. Differences in the NO_2 exposure protocol and species or strain differences may account for these discrepant findings. Our lack of effect on neutrophils and macrophages is in line with this suggestion.

In conclusion, subchronic NO_2 exposure dampened smoke-induced increases in pro-inflammatory cytokines, suggesting an impairment of the immune response. These data correspond with existing literature describing immune modulatory effects of NO_2 exposure, which may lead to increased susceptibility to infections. Future studies are needed to further disentangle the contribution of NO_2 exposure to the inflammatory response in COPD, in particular with respect to susceptibility to infections and exacerbations. Combining NO_2 exposure with an infectious stimulus might result in a pronounced inflammatory response in the lungs, resembling an animal model for COPD exacerbations. Longer exposure durations, as in the human situation, should also be attempted to extend our findings.

Acknowledgements

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Chapter 4

Cigarette Smoke–induced Emphysema
A Role for the B Cell?

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Abstract

Rationale: Little is known about what drives the inflammatory reaction in the development of chronic obstructive lung disease. B cells have been found.
Objective: To study the involvement of B cells in the development of emphysema.
Methods: The presence of B-cell follicles and their interaction with other cells were investigated in lungs of patients with chronic obstructive pulmonary disease and of smoking mice. B cells were isolated from lymphoid follicles by laser microdissection and analyzed for the presence of immunoglobulin rearrangements and somatic mutations.
Main Results: Lymphoid follicles consisting of B cells and follicular dendritic cells with adjacent T cells were demonstrated both in the parenchyma and in bronchial walls of patients with emphysema. A clonal process was observed in all follicles and the presence of ongoing somatic mutations was observed in 75% of the follicles, indicating oligoclonal, antigen-specific proliferation. Similar lymphoid follicles were detected in mice that had developed pulmonary inflammation and progressive alveolar airspace enlargement after smoking. The increase in the number of B-cell follicles was progressive with time and correlated with the increase in mean linear intercept. Specific bacterial or viral nucleic acids could not be detected.
Conclusions: B-cell follicles with an oligoclonal, antigen-specific reaction were found in men and mice with emphysema. In mice, the development was progressive with time and correlated with the increase in airspace enlargement. We hypothesize that these B cells contribute to the inflammatory process and/or the development and perpetuation of emphysema by producing antibodies against either tobacco smoke residues or extracellular matrix components.

Introduction

Although the pathogenesis of chronic obstructive pulmonary disease (COPD) is still not clear, it is obvious that smoking is a major etiologic factor in the Western world. More than 90% of patients with COPD actively smoke or have done so. However, only 15 to 20% of all smokers eventually develop COPD, suggesting an interindividual variability in susceptibility to cigarette smoke (1). Imbalances in proteases/antiproteases, oxidants/antioxidants, or more generally inflammatory/antiinflammatory effects have been postulated to play a role in the development of COPD, but none has been proven to play a pivotal independent role (2,3).

It is widely accepted that the proteolytic potential of neutrophils and macrophages is important for the destruction of the extracellular matrix in emphysema. This is supported by increased numbers of neutrophils and macrophages in both airways and parenchyma of patients with COPD (4,5). Moreover, animal studies have demonstrated that macrophages and their proteolytic activity are a prerequisite for the development of cigarette smoke–induced emphysema (6,7). In addition, Churg and coworkers (8) demonstrated that neutrophil elastase, and thus the neutrophil, is essential for cigarette smoke–induced emphysema in mice.

Little is known about the role lymphocytes play in the development of COPD. Several studies in human tissue sections have demonstrated increased numbers of CD8 and CD4 T cells in the circulation, airways, and parenchyma of patients with COPD (9–12). Even less is known about the role B cells might have in the development of COPD. Bosken and coworkers (13) detected B cells organized in follicle-like structures that were present in airway adventitia of smokers. Hogg and coworkers (12) assessed the inflammation in small airways of surgically resected lung tissue from patients with stage 0–4 COPD according to the severity classification of the Global Initiative for Chronic Obstructive Lung Disease (GOLD) (1). They described the presence of increased numbers of small airways containing CD4 and CD8 lymphocytes, and B cells. As in the earlier work from their group (13), they found a marked increase in lymphoid follicles in the small airways of patients with GOLD stage 3–4 COPD compared with stage 0–2. The role, and thus importance, of these lymphoid follicles remained unclear (12). We hypothesize that B cells contribute to the development of COPD by means of a specific antigen-driven process. Therefore, we studied the presence, organization, and clonality of these cells both in human lung tissue and in our mouse model for cigarette smoke–induced emphysema.

Some of the results of these studies have been previously reported in the form of an abstract (14, 15).

Methods

Subjects

Human lung tissue from surgical resection material of eight patients with COPD (two lung volume reduction surgery, the others lung transplantation) was used (16). COPD was certified by the GOLD lung function criteria (1) and the presence of emphysema was based on histologic examination of lung tissue. The mean

FEV_1 was 23% predicted, the mean FEV_1/VC was 35%. For further details, see the supplement.

Characterization of Lymphoid Follicles in Human Lung Sections

Three-micron-thick paraffin-embedded lung sections were stained by a three-step immunoperoxidase procedure (16). Antibodies were directed against B cells; follicular dendritic cells; CD27 memory B cells; CD3, CD4, and CD8 T cells; CD38 and CD138 (plasma cells); the costimulatory molecule CD40; and proliferation marker Ki-67, which is normally expressed in active germinal centers (for details, see the supplement).

Immunoglobulin Gene Analyses of B-Cell Infiltrates in Human Lungs

CD20-positive B-cell follicles were harvested from 20-m-thick frozen sections by laser microdissection. The dissected cells were captured in polymerase chain reaction (PCR) buffer. The cells were incubated at 60°C for 1 h and protease K was subsequently inactivated by heating at 98°C for 15 min, and stored at –20°C until further use. Immunoglobulin gene analyses were performed by a nested PCR as described previously (17). FR1 and FR3 primers were used. The PCR products were excised from the gel and cloned. The presence of inserts was checked by PCR, using M13 primers. The PCR products were purified, sequenced, and subsequently subjected to alignment analyses with SeqManII and MegAlign software (both from DNASTAR, Madison, WI), and where possible phylogenetic trees were constructed. Twenty-five to 30 clones were analyzed per follicle. For details, see the supplement.

Mouse Smoke Exposure

C57BL/6J mice were exposed to cigarette smoke from 2R1 reference cigarettes twice daily (2 cigarettes/session, 10 puffs/cigarette), 5 d/wk by nose-only exposure (18, 19). The animal experiments were approved by the local ethics board for animal experiments.

Evaluation of Emphysema and Inflammation in Mice

For all details, see the supplement.

After 2, 4, and 6 mo of smoke or sham exposure, the mice were killed. The right lung was snap frozen. The left lung was inflated and fixed with formalin with 25 cm H_2O for 24 h. The mean linear intercept was determined on 25 to 30 photomicroscopic images per animal as a measure of alveolar airspace enlargement by two independent individuals in a blinded manner (20). Inflammatory infiltrates were assessed semiquantitatively on hematoxylin- and eosin-stained paraffin-embedded lung sections. Neutrophils, macrophages, and B cells were detected with anti–GR-1, anti–MAC-3, and anti-B220, respectively, in lung material treated with Nakane's fixative and double stainings were performed on frozen lung sections. The presence of follicular dendritic cells was investigated with FDC-M1 and FDC-M2. Cytokine Analysis in Murine Lung Homogenates The proinflammatory cytokines interleukin 1 (IL-1), tumor necrosis factor, IL-6, and KC (murine homolog of IL-8) and cytokines involved in the differentiation and proliferation of B cells, such as IL-4, IL-6, and IL-13, were measured in a multiplex ELISA system on whole lung homogenates.

Statistics

Mann-Whitney U tests were performed to detect differences between smoking and nonsmoking groups. Differences were considered significant at p 0.05. The analyses were performed with the SPSS version 11 statistical software package (SPSS, Chicago, IL).

Results

Characterization of Lymphoid Follicles in Human Lung Tissue

Lymphoid follicles were detected both in the airway walls and in the parenchyma in lung sections of patients with emphysema (Figure 1). The majority of these follicles (80%) were detected in the parenchyma of the lung rather than in the airway walls. As indicated, follicles in the airway wall were found, but only a minority were within the subepithelial area and thus compatible with bronchus-associated lymphoid tissue (BALT). Changes in the epithelium overlaying the follicles that are indicative of BALT were rarely seen. The majority of cells in the follicles were B cells and follicular dendritic cells were additionally present (Figures 1A–1D). The predominant immunoglobulin expressed on the surface of the B cells was IgM (Figure 1E), whereas cells with other immunoglobulin isotypes were present in low numbers (not shown). The B cells in the infiltrates were predominantly CD27 positive, suggestive of memory B cells (Figure 2A) (21). CD138 plasma cells were detected in the near vicinity of the follicles (data not shown). Almost all cells within follicles expressed the costimulatory molecule CD40 (Figure 2B) on their membrane. In addition, the proliferation marker Ki-67 was found in the lymphoid follicles (Figure 2C) (22). Both CD4 and CD8 T cells were found directly adjacent to the B cells, the majority (80–90%) being CD4 positive.

Immunoglobulin Gene Analyses of B Cells from Infiltrates from Humans

Ten lymphoid follicles were isolated by laser microdissection from the lung tissue of eight patients with COPD. Sequence analysis of the immunoglobulin rearrangements revealed the presence of 12 different B-cell contigs with one or more sequence variations in 10 of the contigs. In seven of eight patients clonal B-cell populations were observed, whereas in one patient only unrelated sequences were observed. The seven patients with related immunoglobulin sequences demonstrated the presence of ongoing mutations (Table 1).

Mouse Model of Cigarette Smoke–induced Emphysema: Airspace Enlargement

A 7% increase in mean linear intercept was observed in smokers as compared with nonsmoking control subjects (p=0.03) after 4mo of cigarette smoke exposure, indicating the onset of emphysema (Figure 3). After 6 mo of smoke exposure the mean linear intercept was increased by 13% (p=0.007).

Characterization of Lymphoid Follicles in Murine Lung Tissue

Histologic evaluation of hematoxylin and eosin staining of paraffin-embedded tissue revealed the presence of pigmented macrophages in lung parenchyma

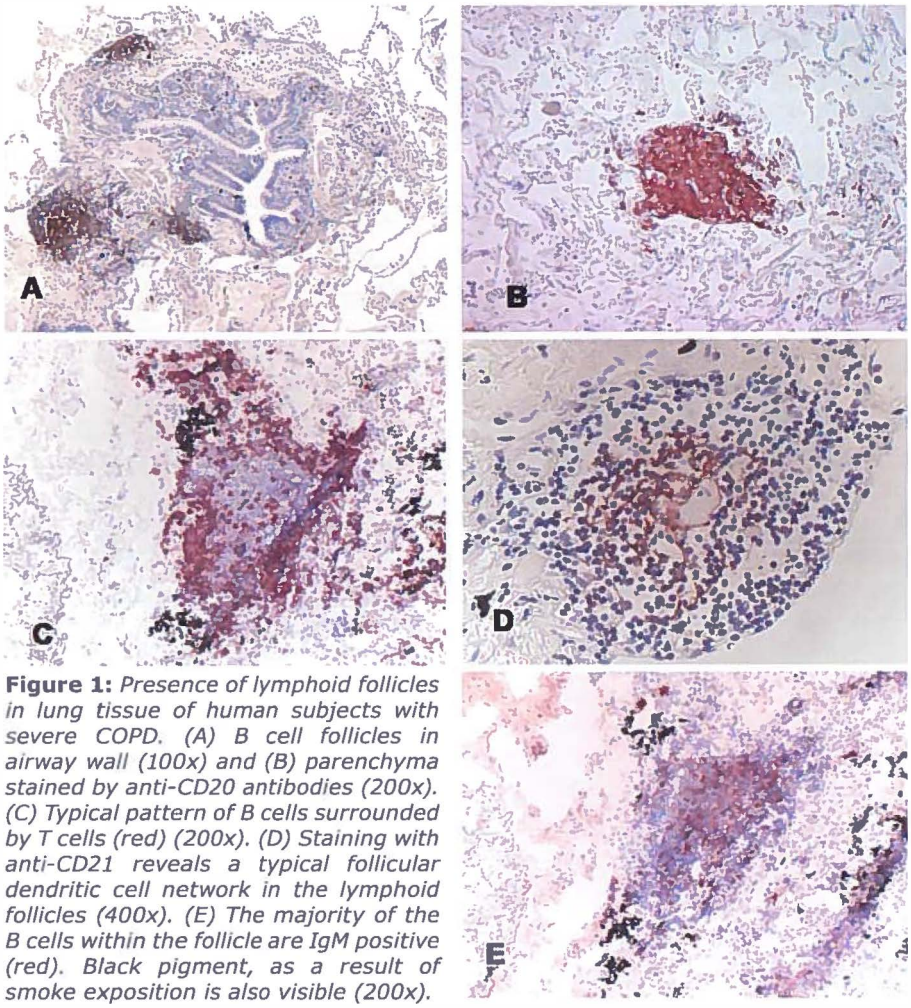


Figure 1: *Presence of lymphoid follicles in lung tissue of human subjects with severe COPD. (A) B cell follicles in airway wall (100x) and (B) parenchyma stained by anti-CD20 antibodies (200x). (C) Typical pattern of B cells surrounded by T cells (red) (200x). (D) Staining with anti-CD21 reveals a typical follicular dendritic cell network in the lymphoid follicles (400x). (E) The majority of the B cells within the follicle are IgM positive (red). Black pigment, as a result of smoke exposition is also visible (200x).*

of smoking mice (Figure 4). Inflammatory infiltrates, with a predominantly lymphoid character, were found to be present both in the parenchyma and around terminal bronchioles (Figure 4). Semiquantitative scoring on tissue sections of smoking mice showed that both the number and the size of these inflammatory infiltrates increased during prolonged exposure to cigarette smoke. The number of infiltrates was significantly higher after 6 mo of smoke exposure than after 2 and 4 mo of smoke exposure (Figure 5).

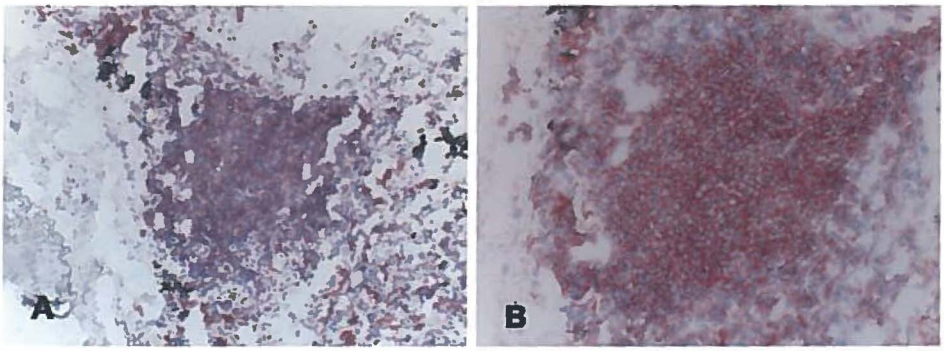
Double staining for CD3 and B220 indicated that the inflammatory infiltrates consisted mainly of B cells surrounded by T cells (Figure 6A). IgM was present on the membranes of these Bcells (Figure 6B), but not IgD, IgG, IgE, and IgA. Macrophages and neutrophils were present in the perimeter of the infiltrates. Only scattered plasma cells were seen in the parenchyma; most were IgM positive and generally not related to the follicular aggregates.

Similar to human emphysematous tissue, the murine B-cell aggregates were lymphoid follicles, given the presence of follicular dendritic cells (Figure 6C). Both CD4 and CD8 T cells were present around the lymphoid follicles, the majority (80%) being CD4 positive (data not shown).

Table 1. Results of Vh gene analyses of B cells harvested by laser dissection from parenchymal follicles in surgical specimens of humans with chronic obstructive pulmonary disease

Patient No.	No. Follicles	No. Clones Sequenced	Single Sequences	No. B-cell Contigs (No. Clones per Contig)	No. Sequence Variants per Contig (No. Sequences per Variant)	Ongoing Mutations
1	1	32	17	2 (9 and 6 seq)	2 variants (5/4)	Yes
					2 variants (4/2)	Yes
2	1	29	10	1 (19 seq)	6 variants (3/4/3/2/5/2)	Yes
3	1	27	6	3 (6, 9, and 6 seq)	1 variant (6)	Yes
					3 variants (5/3/1)	No
					4 variants (2/2/1/1)	Yes
4	2	27	2	1 (25 seq)	5 variants (14/3/1/4/3)	Yes
		26	10	2 (10 and 6 seq)	3 variants (4/4/2)	Yes
					6 variants (1/1/1/1/1/1)	Yes
6	2	25	12	1 (13 seq)	4 variants (9/2/1/1)	Yes
		28	0	1 (28 seq)	7 variants (10/3/2/3/2/3/5)	Yes
7	1	9	2	1 (7 seq)	1 variant (7)	No
8	1	24	24	0	—	No

Definition of abbreviation: seq = sequences.



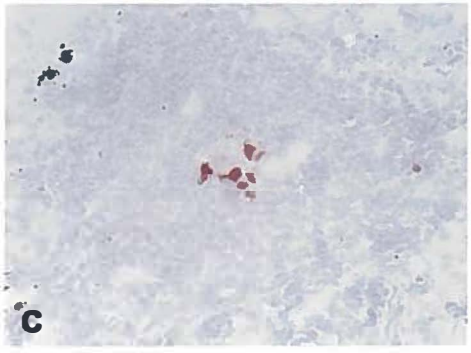


Figure 2: *Immunohistochemical characterization of B cells in lymphoid follicles in lung tissue of COPD patients. (A) the majority of the lymphocytes are CD27⁺ memory B cells (200x). (B) The co-stimulatory marker CD40 is present on nearly all cells within the lymphoid follicle (400x). (C) Ki-67 antigen is expressed in the center of the lymphoid follicle (100x).*

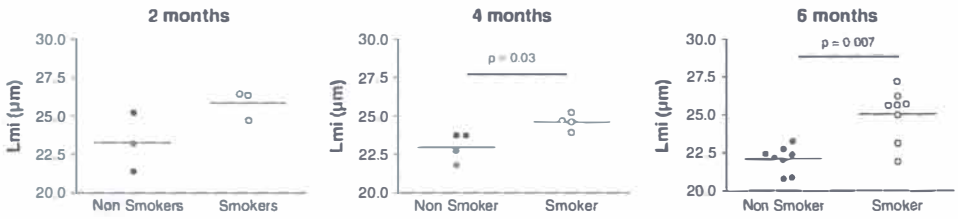


Figure 3: *Time course of change in mean linear intercept due to cigarette smoke exposure in mice. From 4 months cigarette smoke exposure, mean linear intercepts are significantly increased in smoking mice compared to non-smoking mice.*

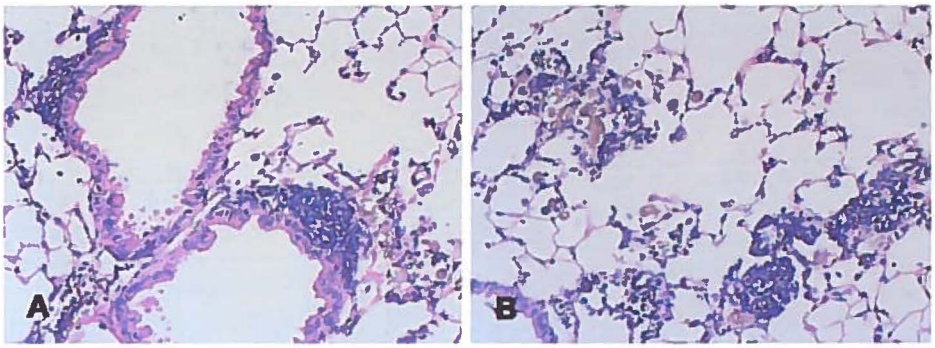


Figure 4: *Pulmonary inflammation after cigarette smoke exposure in mice. Pigmented macrophages and inflammatory infiltrates can be observed, both (A) around the bronchioles and (B) in the parenchyma (both 100x).*

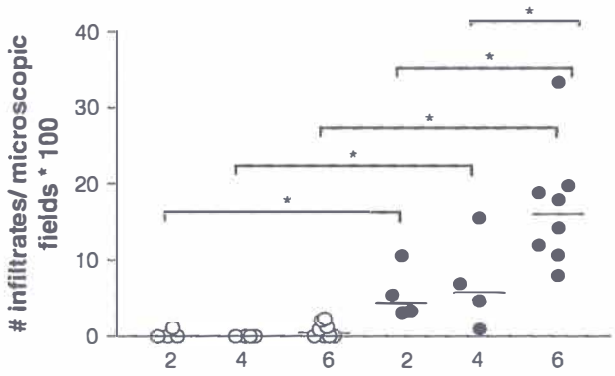


Figure 5: *Semiquantitative assessment of pulmonary inflammation after smoke exposure in mice. The number of inflammatory infiltrates is increased in smoking mice (o) compared to non-smoking mice (•) at all time points. Within the smoking mice group, there is also a significant increase at later time points compared to earlier time points. *: $p < 0.05$*

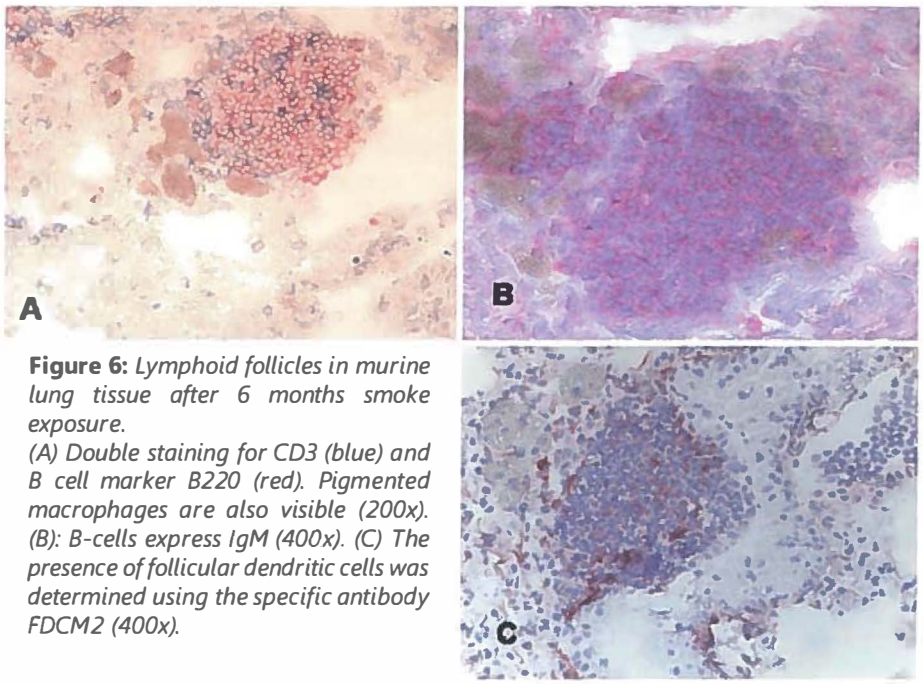


Figure 6: *Lymphoid follicles in murine lung tissue after 6 months smoke exposure.*
(A) Double staining for CD3 (blue) and B cell marker B220 (red). Pigmented macrophages are also visible (200x). (B): B-cells express IgM (400x). (C) The presence of follicular dendritic cells was determined using the specific antibody FDCM2 (400x).

Cytokine Analyses on Murine Lung Homogenates

Significantly higher levels of the proinflammatory cytokines IL-1, tumor necrosis factor-α, IL-6, and KC (murine IL-8) were found after 4 mo of smoking (Figure 7). IL-6, IL-4, and IL-13 are all thought to play a role in B-cell proliferation, and were elevated.

Discussion

Our data show the presence of lymphoid aggregates in the airways and in the lung parenchyma of humans with emphysema. The aggregates consisted mainly of B cells interspaced by follicular dendritic cells and surrounded by T cells. Sequence variations in the individual B-cell contigs were demonstrated in the B cells, indicating oligoclonal B-cell proliferation in response to stimulation with as yet unknown antigen(s) (22).

B-cell follicles have been demonstrated previously in the small airways of patients with COPD by the group of Hogg and coworkers, both more recently (12) and in earlier work (13). Our findings are novel in several ways. First, we assessed not only the small airways but also the parenchyma. We found that more than 80% of the follicles were located in the parenchyma. This is much more than can be attributed only to effects of airways being adjacent to (either directly above or below) the sampled section. Furthermore, the composition of the B-cell follicles was analyzed: the B-cell follicle core was surrounded by T cells, the majority being CD4 positive. The B cells were interspaced by follicular dendritic cells that are necessary for antigen presentation and affinity maturation.

Expression of Ki-67 antigen and the costimulatory molecule CD40 indicates local blast transformation compatible with an (early) germinal center reaction (22). In addition, sequence analysis of rearranged immunoglobulin genes in individual B-cell clones harvested from follicles by laser microdissection revealed the presence of ongoing mutations in clonally related B cells. Together, these data indicate (oligo)clonal B-cell proliferation and support a true germinal center reaction.
Interestingly, analogous to our findings in humans with COPD, similar lymphoid follicles were observed in lung tissue in our smoking mouse model. This model produces progressive emphysema over time. Starting at 4 mo of exposure, a progressive increase in both size and number of these follicles was found. As in humans, the follicles contained B cells surrounded by CD4 and CD8 T cells and additionally centrally located follicular dendritic cells.

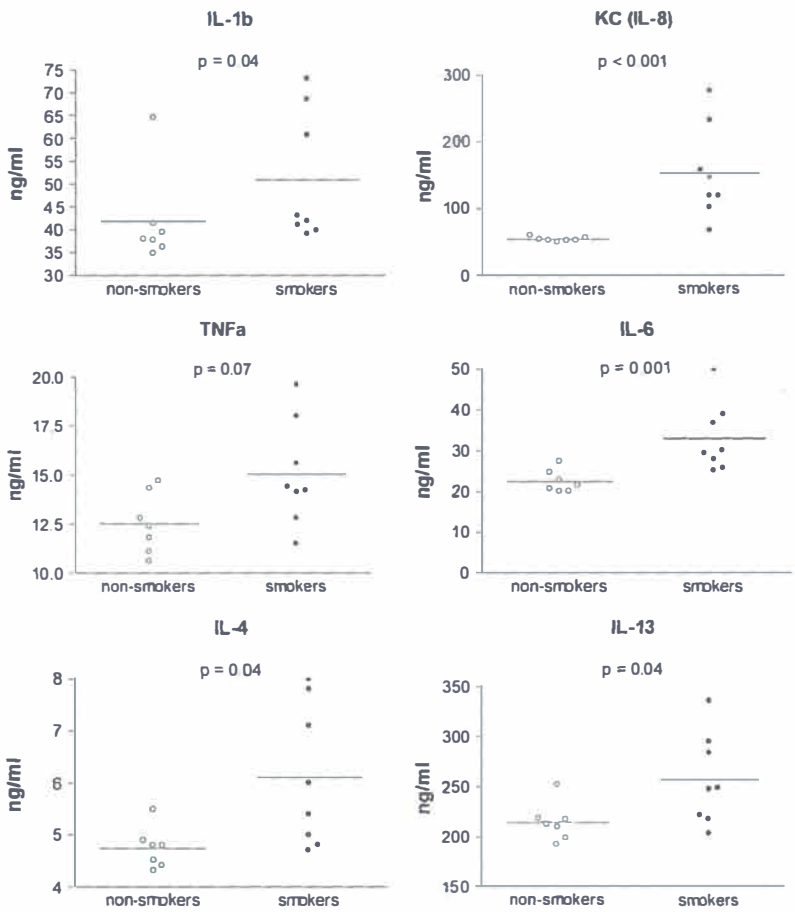


Figure 7: *Inflammatory cytokines and chemokines in murine lung homogenates after 4 months smoke exposure. Asterisks (*) indicate statistically significant differences in chemokine/cytokine concentrations between smoking animals and controls.*

We found associations between follicle formation and increased levels of cytokine that are involved in B-cell proliferation. IL-4, IL-6, KC (IL-8), and IL-13 were observed in the lung tissue homogenates of our smoking mice. These cytokines are essential for the formation and differentiation of germinal centers (23). Overexpression of IL-6 has been shown to give rise to areas of proliferating B-cell follicles in mice (24). In addition, overexpression of IL-13 in mice results in severe emphysema (25).

Thus, the presence of B-cell follicles has now been demonstrated in human and murine lung tissue in association with emphysema, and in both the airway wall and lung parenchyma. The important question that arises from these findings is the potential role of these B cells in the development of emphysema. The observed ongoing mutations in clonally relatedBcells within the lymphoid follicles suggest an antigen-driven selection process. At present, it is unclear against which antigen(s) this B-cell proliferation is directed. We would like to put forward the following speculative but tempting considerations. At least three potential sources of antigens should be considered: microbial, cigarette smoke components or derivatives, and degradation products of extracellular matrix. Part of the reactivity of the observed B cells may be directed against bacterial or viral antigens. Indeed, most patients with COPD are colonized with bacteria (26). In particular, adenovirus has been implied in COPD (27,28) and B cells may play a role in this respect, even though the major part of an antiviral response is of a cellular (cytotoxic) nature (29). Hogg and coworkers have demonstrated B-cell follicles in the small airways of humans with emphysema that were labeled as BALT because of their subepithelial localization. The authors expressed the expectation that the B-cell follicles represented a response against microbial antigens (12,30), but did not include further support. In our study, the majority of B-cell follicles were not seen within the subepithelial area. The few follicles we observed in relation to the airways were not covered by recognizable lymphoepithelium or M cells that transport antigens across the epithelium and are specific to BALT (31). Moreover, the littermate control mice we used in our studies were exposed to the same housing and (sham) handling, but did not develop these follicles. Analysis of fresh frozen tissue samples by real-time PCR did not indicate any presence of mycoplasma, chlamydia, adenovirus, or Pneumocystis jiroveci. Real-time PCR analysis of these samples using broad-range primers reactive with 16S RNAs from prokaryotes did not show any reactivity above baseline levels, thus providing no evidence for the presence of specific bacterial pathogens. A second source of antigens that could theoretically cause specific B-cell proliferation is cigarette smoke. Cigarette smoke contains approximately 4,500 different compounds (31), of which some are proteins and therefore potentially immunogenic (32). Some of these compounds will precipitate in the lung, possibly bind to the extracellular matrix, and may elicit an antibody response. Alternatively, reactive components from smoke can react with proteins in the tissue to form new, immunogenic protein adducts (33). Subsequently, immune complex formation may occur, eliciting an inflammatory response and subsequently tissue degradation.

Finally, the extracellular matrix (ECM) itself may be a source of antigens. Earlier studies have demonstrated that breakdown products of several ECM proteins such as hyaluronic acid, elastin, and collagen have chemotactic and activating

effects on neutrophils and macrophages, resulting in the release of oxidants and proteases that are detrimental to the ECM (34,35). In addition, hyaluronic acid causes activation and proliferation of B cells (36). Apart from this general chemotactic and activating role, we hypothesize that cigarette smoke–induced breakdown products of the ECM might additionally be immunogenic and trigger a specific B-cell reaction. The induced anti-ECM antibodies may subsequently bind to fragments of, or to intact, ECM, causing further degradation of ECM by phagocytes. This would also be compatible with our observation that the immunoglobulins we found were of the IgM isotype because these can be involved in antibody reaction to extracellular matrix products, especially polysaccharides (37). The response to polysaccharides is often T-cell independent in mice and related in particular to the B1 B-cell (IgM and IgD) CD5 subset, whereas in humans this involves the (spleen-based) marginal zone B-cell subset (IgM and IgD–, strongly CD21, and CD5–). The lung B cells in our study were CD5– and weakly CD21 in humans, and also CD5– in mice (our unpublished results). The absence of IgD may thus indicate an activated state of lung B cells, but does not indicate a specific origin or nature. Moreover, the fact that it was already known that CD8 and CD4 T cells are prominent in COPD enables the suggestion that CD4 cells in particular may result from nonspecific activation and provide specific help to cytotoxic CD8 cells and for T-cell dependent B-cell responses.

Our reasoning regarding B cells in the lung is speculative, yet it is supported by the presence of similar B-cell follicles in the inflamed synovia and a humoral response against ECM fragments that has been documented in rheumatoid arthritis (38–40). It is conceivable that a viral or bacterial infection or colonization, as frequently seen in COPD, could lead to breakdown of tolerance, facilitating such a reaction against selfantigens. Such events are thought to play a role also in the initial phase and during exacerbations of several autoimmune diseases (41).

Both mechanisms described above, that is, smoke- or ECM derived antigens as inductors of an inflammatory process, may also explain the observation that pulmonary inflammation continues in patients with COPD after smoking cessation (42). We did not investigate the effects of smoking cessation in this mouse model. It is well known that at least some tobacco residues remain in the lung for a long time and thus may maintain inflammation of the airways and lung parenchyma. Direct or indirect antibody-mediated degradation of ECM results in newly formed ECM fragments, which in turn may contribute to perpetuation of the inflammatory reaction. These findings and considerations regarding the antigen specificity of the B-cell response are also compatible with a hypothesis that an autoimmune component may play a role in the development or perpetuation of COPD (43, 44), in which, therefore, besides CD4 and CD8 T cells, B cells may also be implicated.

In summary, we demonstrate the presence of B-cell follicles in the parenchyma of patients with emphysema. Immunoglobulin gene analysis revealed an oligoclonal process with ongoing mutations, suggesting an antigen-driven process that is probably not of microbial origin. Putative roles for these B cells include a reaction to cigarette smoke components or to extracellular matrix degradation products. The presence of similar B-cell follicles in a smoking mouse model opens additional

avenues for further research into the exact role of B cells in the pathogenesis of emphysema.

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Online data supplement

Methods

Subjects

Human lung tissue from our bank of surgical resection material of patients with COPD were used [1]. COPD was certified by the combination of forced expiratory flow in one second (FEV_1) and FEV_1/ forced vital capacity (FVC) as per GOLD criteria [2] and the presence of emphysema was based on histological examination of lung tissue performed by an experienced pulmonary pathologist (WT). When resection had been carried out for tumors, only lung tissue distant from the tumor was included. Tissue of the severe emphysema group was obtained from lung resections of patients without α1-antitrypsin deficiency who underwent single or double lung transplantation or lung volume reduction surgery.

Immunohistochemical characterization of lymphoid follicles in human lung sections

Three-micron thick paraffin embedded lung tissue sections were stained by three step immunoperoxidase procedure with amino-ethyl-carbazole (AEC, Sigma Aldrich, Zwijndrecht, The Netherlands) as the chromogen, as described previously [1]. The specific antibodies and their sources are presented in Table E1. Antibodies were directed against B cells, follicular dendritic cells, $CD27^+$ memory B cells, $CD3^+$, $CD4^+$, and $CD8^+$ T-cells. CD38 and CD138 antibodies were used to detect plasma cells, and CD40 antibodies to detect the presence of this co-stimulatory molecule. Antibodies against ki-67 were used to detect this proliferation marker which is normally expressed in parts of follicular germinal center cells. The expression of immunoglobulins (Ig) in lymphoid follicles was investigated according to a standard, three-step indirect staining method, see also Table E1.

Immunoglobulin-gene analyses of B cell infiltrates in human lungs

Frozen human lung tissue sections of 20 µm thick were stained for B cells (CD20) and the immunopositive follicles were subsequently harvested using a laser-microdissecting microscope (Palm, Bernried, Germany). The micro dissected cells were captured in 30µl polymerase chain reaction (PCR) buffer (RB, Pharmacia, Roosendaal, The Netherlands) with the addition of 1µl Protease K per vial. The cells were incubated at 60°C for 1 h to purify the DNA and protease K was subsequently inactivated by heating at 98°C for 15 min. The resulting DNA solutions were stored at –20°C until further use.
Immunoglobulin-gene analyses were performed using a nested PCR as described previously [3]. Primers located in frame work region 1 and 3 (FR1 and FR3) of the immunoglobulin heavy chain gene were used in combination with a J-segment primer in the first and in the second amplification round, respectively. The PCR products were analyzed on a 4% agarose gel (Biozym). PCR products (± 120 bp) were excised from the gel, purified, and cloned

in the pCR2.1 vector using the TopoII-cloning kit (Invitrogen, Breda, The Netherlands). The presence of inserts was checked by PCR using M13-primers, which flank the multiple cloning site of the pCR2.1 vector. The PCR products were purified, sequenced, and subsequently subjected to alignment analyses using SeqManIITM and MegAlignTM software (DNA star, Madison, WI, USA), and where possible phylogenetic trees were constructed. Approximately 25-30 clones, obtained from one follicle, were purified and analyzed from each patient, see Table 1 in main text.

Animals

Specified pathogen free male C57Bl/6j mice were bought from Harlan, Zeist, The Netherlands and kept at the Central Animal Facility of the Groningen University. The animals received standard rodent food (Hope Farms, Woerden, The Netherlands) and water ad libitum. The animal experiments were approved by the local ethical board for animal experiments.

Smoke exposure

Mice were exposed to cigarette smoke from 2R1 reference cigarettes from the University of Kentucky (Kentucky Tobacco Research and Development Center, formerly the Tobacco Health Research Institute, Lexington, KY). The animals were placed in a restrainer in the smoking machine (Kentucky Tobacco Research and Development Center) and subjected to cigarette smoke by their nose only [4,5]. Smoke was administered twice daily (2 cigarettes per session, 10 puffs per cigarette), 5 days per week. Non-smoking mice were also placed in the restrainers and exposed to room air with the same schedule. The smoking machine was checked for the delivery of total particulate matter as described by Griffith et al [4,5] and calibrated before every smoking session to ensure accurate and standardized smoke delivery. Actual smoke exposure was assessed by measuring carboxyhemoglobin levels. These ranged from 15-35 % immediately after smoke administration.

Morphometrical evaluation of emphysema

After 2, 4, and 6 months smoke or sham exposure, the mice were anaesthetized with a mixture of isoflurane, N_2O, and oxygen and their trachea was cannulated. Subsequently, the mice were exsanguinated via the abdominal aorta. The right lung was ligated, lung lobes were taken out and either snap frozen or fixed in paraformaldehyde and an increasing concentration up to 20% sucrose buffer with 10% glycerine was added, according to Nakane's method for immunohistochemical evaluation [6]. After this fixation, tissue was snap frozen at –80°C in isopentane and stored at –80°C until further use. The left lung was removed, inflated, and fixed with formalin with a constant pressure of 25 cm H_2O for 24 h. Subsequently, the lung was dissected from the trachea and embedded in paraffin. Three-micron paraffin sections were cut and stained with hematoxylin and eosin. Approximately 25-30 photomicroscopic images per tissue section were prepared at 10x20 magnification using a standardized sequence of image capturing. Images with large vessels, conducting airways or pleura occupying 25% or more of the total image were not used for the

mean linear intercept analysis which was assessed as a measure of alveolar airspace enlargement by two independent individuals in a blinded manner [7].

Quantification and characterization of lymphoid follicles in murine lung tissue

The number and size of inflammatory infiltrates in murine lung tissues was assessed in a semiquantitative manner on hematoxylin and eosin stained paraffin embedded lung sections. The lung sections of all animals were evaluated in at least 30 microscopic fields per animal in a standardized sequence at 10x20 magnification. The infiltrates were scored, based on the size and number of the inflammatory infiltrates. The summed score per animal was divided by the number of microscopic fields assessed. Tissue sections (4 μm) from either snap frozen or Nakane-fixed lung tissue were prepared. Nakane fixation was used in order to obtain a better morphology. However, some antibodies cannot be used on Nakane-fixed tissue and in those cases, snap frozen tissue was used instead. Specific monoclonal antibodies GR-1, MAC-3 and B220 (BD Pharmingen) were used on Nakanefixed lung sections to detect the presence of neutrophils, macrophages and B cells respectively. An immuno alkaline phosphatase procedure was used with fast red RB salt as the chromogen. Double stainings were performed on frozen lung sections to detect $CD3^+/CD4^+$ and $CD3^+/CD8^+$ T cells (see Table E1). In addition, a double staining with CD3/B220 was performed on frozen lung sections to distinguish T cells from B cells. Anti-CD19 was used on frozen lungs as a control for the detection of B cells, since it is known that B220, besides B cells, also stains a subpopulation of antigen presenting cells [8]. Specific antibodies directed against IgD, IgM, IgG, IgA and IgE were applied on frozen lung sections. The presence of follicular dendritic cells was investigated using FDCM1 and FDCM2. For data on the antibodies and techniques use, see Table E1.

Cytokine analysis in murine lung homogenates

Lung homogenates were prepared from female C57Bl/6j mice that were exposed to cigarette smoke for 4 months. Lung tissue was homogenized in (50 mM Tris-HCl buffer, containing 150 mM NaCl, and 0.002% Tween-20, pH 7.5) in 10% w/v. Supernatants were stored at -80° C until analyzed. The concentrations of the pro-inflammatory cytokines Il-1β TNFα, IL-6, and KC (murine homologue of IL-8) and cytokines that are known to be involved in the differentiation and proliferation of B cells such as IL-4, IL-6, and IL-13, were measured in a multiplex ELISA system (Lincoplex Systems, St Charles, MO, USA).

Statistics

Mann-Whitney U tests were performed to detect differences between smoking and nonsmoking groups. Differences were considered significant at $p< 0.05$.

Results

Subjects

Human lung tissue from surgical resection material of patients with COPD were used as described previously [E1]. They were collected consecutively from our tissue bank provided the amount of material was sufficient for analysis. Two patients had received lung volume reduction surgery, the remainder lung transplantation. COPD was certified by the GOLD lung function criteria [E2]. The mean age was 56, all were ex-smokers, with a mean of 38 packyears, the FEV_1 was 23%predicted, and the mean FEV_1/VC was 35%. The presence of emphysema was based on histological examination of lung tissue by WT.

Table E1: Sources of antibodies used

First antibody	Source	Against	Supplier
Human lung tissue			
anti-CD20	mouse	B-cells	Dako, Glostrup, Denmark
anti-CD27	mouse	Memory B-cells	BD Pharmingen, Alphen a/d Rijn, NL
anti-CD38	mouse	Plasma cells	BD Pharmingen, Alphen a/d Rijn, NL
anti-CD138	mouse	Plasma cells	IQ products, Groningen, NL
anti-CD21	mouse	Follicular dendritic cells	Dako, Glostrup, Denmark
anti-CD35	mouse	Follicular dendritic cells	Sanbio, Uden, NL
anti-CD40	mouse	Co-stimulatory molecule	Immunotech, Marseille, France
anti-kI67	mouse	Proliferation marker	BD Pharmingen, Alphen a/d Rijn, NL
anti-CD3	rabbit	T-cells	Dako, Glostrup, Denmark
anti-CD4	mouse	T-helper cells	BD Pharmingen, Alphen a/d Rijn, NL
anti-CD8	mouse	T-cytotoxic cells	Dako, Glostrup, Denmark
anti-IgA	mouse	Immunoglobulin A	Dako, Glostrup, Denmark
anti-IgD	mouse	Immunoglobulin D	BD Pharmingen, Alphen a/d Rijn, NL
anti-IgE	rabbit	Immunoglobulin E	Dako, Glostrup, Denmark
anti-IgG	rabbit	Immunoglobulin G	Dako, Glostrup, Denmark
anti-IgM	mouse	Immunoglobulin M	BD Pharmingen, Alphen a/d Rijn, NL
Murine lung tissue			
anti-B220	rat	B-cells, APC's	BD Pharmingen, Alphen a/d Rijn, NL
anti-CD19	rat	B-cells	BD Pharmingen, Alphen a/d Rijn, NL
FDCm1	rat	Follicular dendritic cells	BD Pharmingen, Alphen a/d Rijn, NL
FDCm2	rat	Follicular dendritic cells	BD Pharmingen, Alphen a/d Rijn, NL
anti-CD3	hamster	T-cells	BD Pharmingen, Alphen a/d Rijn, NL
anti-CD4	rat	T-helper cells	BD Pharmingen, Alphen a/d Rijn, NL
anti-CD8	rat	T-cytotoxic cells	BD Pharmingen, Alphen a/d Rijn, NL
anti-GR-1	rat	neutrophils	BD Pharmingen, Alphen a/d Rijn, NL
anti-Mac3	rat	macrophages	BD Pharmingen, Alphen a/d Rijn, NL
IgA	goat	immunoglobulin A	BD Pharmingen, Alphen a/d Rijn, NL
IgD	goat	immunoglobulin D	BD Pharmingen, Alphen a/d Rijn, NL
IgE	rat	immunoglobulin E	BD Pharmingen, Alphen a/d Rijn, NL
IgG	goat	immunoglobulin G	SBA, Uthoorn, NL
IgM	goat	immunoglobulin M	SBA, Uthoorn, NL

Second antibody	Source	Against	Supplier
RaMpo	rabbit	mouse	Dako, Glostrup, Denmark
GaRpo	goat	rabbit	Dako, Glostrup, Denmark
RaGpo	rabbit	goat	Dako, Glostrup, Denmark
RaMbio	rabbit	mouse	Dako, Glostrup, Denmark
GaRbio	goat	rabbit	Dako, Glostrup, Denmark
GaRatpo	goat	rat	SBA, Uthoorn, NL
GaRatbio	goat	rat	SBA, Uthoorn, NL
MaHamsterbio	mouse	hamster	BD Pharmingen, Alphen a/d Rijn, NL

po = conjugated to peroxidase
bio = conjugated to biotin

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4

Chapter 5

Regulatory T cells and B cells in relation to smoking and COPD

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Submitted

Abstract

Specific immunity plays a role in COPD pathogenesis, since B-cell follicles are present in lung tissue and increased anti-elastin titers in plasma of COPD patients. Additionally, regulatory T cells (Tregs) have been implicated in its pathogenesis as they control immunological reactions. We investigated the presence of Tregs and B cells in peripheral blood of COPD patients and healthy individuals in relation to their current smoking status.

Peripheral blood mononuclear cells obtained from 20 COPD patients and 29 healthy individuals were analyzed for CD4, CD25, Foxp3, HO-1, CD20, CD27, and IgM expression using flow cytometry.

COPD patients had higher Treg percentages and lower B cell percentages in peripheral blood than healthy individuals, with a significant correlation between these cells. Interestingly, current smokers had higher percentages of (class-switched) memory B cells in peripheral blood than ex-smokers and never smokers irrespective of COPD.

The increase in (class-switched) memory B cells in current smokers is intriguing and suggests that smoke-induced neo-antigens are constantly induced in the lung. The negative correlation between Tregs and B cells corroborates previously published observations that Tregs can suppress B cells. Finally, an altered specific immune response in COPD may contribute to the development and/or persistence of the inflammatory response.

Introduction

COPD is a leading cause of death worldwide and its morbidity and mortality are still rising. Although the pathogenesis of the disease is still not fully defined, tobacco smoke is widely accepted as the most important cause for the development of the disease. Until now, the only effective treatment to stop the accelerated lung function decline that is present in COPD is smoking cessation, even though the inflammatory response may persist [1]. More information is needed about the origins and nature of the chronic inflammatory response in COPD to find better treatment targets for COPD patients and to understand why this chronic inflammatory response persists after stopping smoking.

The role of the innate immune response, i.e. neutrophils and macrophages is well established in COPD [2], yet the role of specific immunity, i.e. CD4 T cells and B cells has only recently attracted attention. We and others have found both oligoclonal T- and B cells in the lungs of COPD patients suggesting an antigen driven immune response [3,4]. Additionally, an increased number of small airways containing B cells and lymphoid follicles has been shown to be present in patients with GOLD stage III-IV compared to stage 0-II [5]. Finally, an increase of B cells in the mucosa of large airways was demonstrated in COPD patients compared to controls [6].

An important modulator of the immune system is the regulatory T cell (Treg). Tregs are a special subset of $CD4^+$ T lymphocytes, which is important in controlling immunological tolerance and preventing auto-immune reactions by inhibiting T-cell responses [7]. Tregs can also directly inhibit B-cell responses, by suppressing class switch recombination and Ig production in B cell follicles without requiring suppression of adjacent T cells [8,9]. Dysfunction of Tregs has been shown to contribute to auto-immune diseases, allergy, and chronic inflammatory diseases [7,10]. The currently best described subset of Tregs is that of the naturally occurring Tregs, which express high levels of CD25 and Forkhead transcription factor 3 (Foxp3). The suppressive effects of Tregs are mediated by cell-cell contact and possibly also by heme oxygenase-1 (HO-1) expression and membrane bound transforming growth factor (TGF)-β [11-13]. Three recent studies have investigated the presence of Tregs in COPD, but they reported different findings in lung tissue and bronchoalveolar lavage (BAL). The first study showed decreased numbers of $CD4^+CD25^+$ Tregs and Foxp3 mRNA levels in lung tissue of emphysema patients compared to control subjects [14]. The second showed increased numbers of $CD4^+CD25^{bright}$ Tregs in BAL from COPD patients and healthy smokers when compared to healthy never smokers [15], while the third study showed decreased $CD4^+CD25^+$ Tregs in BAL of COPD patients and never smokers compared to healthy smokers [16]. In all three studies, the Treg presence was analyzed by measuring $CD4^+CD25^+$ T cells. Foxp3 was used in separate analyses to prove that a high percentage of these $CD4^+CD25^+$ T cells were positive for Foxp3 and thus Tregs. This way of analyzing Tregs could explain the discrepant findings, since CD25 expression varies with the activation state of T cells, which might differ between lung and BAL T cells. None of these studies found differences in $CD4^+CD25^+$ Tregs in peripheral blood of COPD patients and healthy individuals.

We hypothesize that the B-cell reaction in COPD is antigen driven and contributes to progression of the disease, and that Tregs are involved in the suppression of this B cell mediated response. In order to find support for this hypothesis we investigated the presence of both (memory) B cells and Tregs, together with HO-1 expression in peripheral blood mononuclear cells obtained from COPD patients and healthy volunteers. In comparison to the above mentioned studies, we defined Tregs by co-expression of CD4, CD25 and Foxp3, and analyzed the percentage of Foxp3 expressing $CD4^+CD25^+$ T cells. In order to disentangle separate effects of COPD and current smoking status on the presence of the different cell types, equal numbers of COPD smokers and ex-smokers as well as healthy smokers, ex-smokers and never smokers were recruited for this study.

Materials and Methods

Subjects

COPD patients and healthy individuals were recruited to participate in this study. Inclusion criteria for COPD patients were; clinical diagnosis of COPD, postbronchodilator FEV_1 < 80% predicted, postbronchodilator FEV_1/FVC < 70%, and no exacerbation in the 6 weeks preceding the study. Inclusion criteria for healthy individuals were; no signs and symptoms of pulmonary disease, FEV_1 > 90% predicted, and FEV_1/FVC > 70%.

All participants met the following criteria: age > 40 years, negative skin prick tests for the most common aeroallergens, no use of (inhaled) corticosteroids in the 6 weeks preceding the study, and no comorbidities. To avoid the effect of gender only males were included in the study. Smokers and ex-smokers had to have a smoking history of at least 10 packyears and ex-smokers had to have quit smoking for a least one year. The medical ethics committee of the University Medical Center Groningen approved the study and all participants gave their written informed consent.

Flow cytometry analysis

All participants donated 20 ml of peripheral blood. Peripheral blood mononuclear cells (PBMCs) were isolated using ficoll-paque plus (GE Healthcare, UK) density gradient centrifugation. Total isolated cells were counted using a Sysmex pocH-100i cell counter (Sysmex, Roche, Germany).

Two antibody cocktails were used to stain PBMCs for 1) Tregs and 2) B cells.

1. CD4-AmCyan (BD Biosciences, San Jose, USA), CD25-Pe-Cy7 (eBioscience, San Diego, USA), Foxp3-Alexa Fluor 700 (eBioscience), and HO-1-FITC (StressGen, Victoria, Canada)
2. CD20-PE-Cy5, CD27-FITC, and IgM-biotin followed by Streptavidin-PE (all BD Biosciences).

Appropriate isotype controls were used for the CD25 (mouse IgG1-Pe-Cy7, eBioscience) and Foxp3 (rat IgG2a-Alexa Fluor 700, eBioscience) staining.

To stain for surface markers, 1*106 cells were first incubated for 15 minutes with 0.5% human serum (Sigma-Aldrich, Zwijndrecht, the Netherlands), and then centrifuged and incubated with the appropriate antibody cocktail for 30

minutes on ice, protected from light. After washing the cells of both cocktails with phosphate buffered saline solution (PBS) supplemented with 2% bovine serum albumine (BSA, Serva, Heidelberg, Germany), the cells of cocktail 2 were incubated for 15 minutes with Streptavidin-PE, washed three times with PBS/2%BSA, resuspended in FACS lysing solution (BD Biosciences), and kept in the dark on ice until flow cytometry analysis. The cells of cocktail 1 were fixed and permeabilized for 30 minutes using a fixation and permeabilization buffer kit (eBioscience), and then washed with permeabilization buffer, blocked with 2% human serum and then incubated with anti-Foxp3 for 1 hour. Afterwards the cells were washed with permeabilization buffer, resuspended in FACS lysing solution, and kept in the dark on ice until flow cytometric analysis. The fluorescent staining of the cells was measured on a LSR-II flowcytometer (BD Biosciences) and data were analyzed using FlowJo Software (Tree Star, Ashland, USA).

Statistical analysis

A multiple linear regression model was used to establish whether there was a significant interaction between the presence of COPD and current smoking status for the different Treg and B cell parameters. The model was tested with the effects of COPD, current smoking, and with the interaction between COPD and current smoking as independent variables. The normal distribution of the residuals was analyzed with a Kolmogorov-Smirnov test and when needed the data were log-transformed to normalize distributions. When the interaction between COPD and current smoking was not significant, Mann Whitney U tests were used to establish differences between the groups. The relation between B cells and CD4+CD25+Foxp3+ T cells was evaluated with the Spearman correlation. A value of $p<0.05$ was considered significant.

Results

Patient characteristics

The characteristics of the twenty COPD patients (current and ex-smokers) and twenty-nine healthy volunteers (current, ex- and never smokers), included in the study, are shown in table 1. COPD patients were slightly older than the healthy individuals, which was mainly caused by the younger age of the healthy smokers. Additionally, COPD patients had more packyears of smoking when compared to healthy current and ex-smokers. One healthy "never smoker" was included who had a smoking history of 2.5 packyears and had stopped smoking for 40 years, the other never smokers had no smoking history at all.

Regulatory T cells in peripheral blood

Tregs were defined as $CD4^{+}CD25^{+}Foxp3^{+}$ T cells. The positive gates for CD25 and Foxp3 expression were based on the expression levels of the appropriate isotype controls, and a separate $CD25^{high}$ gate was set on the high population (Figure 1).

Table 1: Characteristics of COPD patients and healthy individuals

	COPD patients		Healthy individuals		
	Current smokers	Ex-smokers	Current smokers	Ex-smokers	Never smokers
Subjects (n)	10	10	9	10	10
Age (years)	65.9 (4.3) *	66.7 (7.4) *	52.8 (4.1) #	61.1 (9.3)	58.1 (6.5)
Packyears	34 (13.5) *	36.7 (18.2) *	24.6 (11)	20.6 (5.9)	0.3 (0.8)
FEV_1 post BD (% pred.)	44.9 (14.9) $	60.7 (14.7)	105.6 (8.7)	115.7 (15.9)	111.1 (12.1)
FEV1/FVC post BD, (%)	37.6 (10)	43.8 (10.7)	76.4 (4)	78.2 (5.9)	78.5 (4.1)

Mean (standard deviation) is depicted. Mann Whitney U tests were used to test differences between the groups. FEV_1= Forced expiratory volume in 1 second. FVC= Forced vital capacity. BD= Bronchodilator.
** COPD patients versus healthy individuals p < 0.05*
 # Healthy current smokers versus healthy ex- and never smokers p< 0.05
$ COPD smokers versus COPD ex-smokers p < 0.05

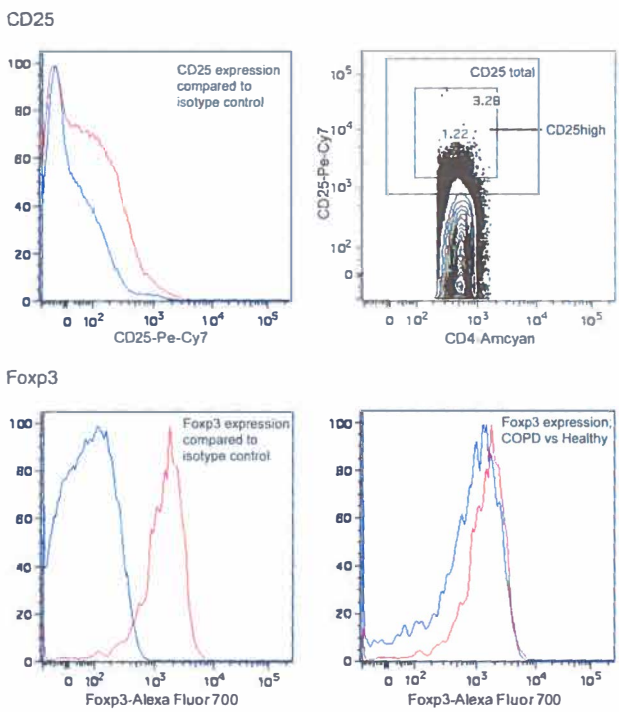


Figure 1. Flow cytometry plots of regulatory T cells in peripheral blood.
The CD25 expression (red curve) compared to the isotype (blue curve), and the CD25 total and CD25high gates are depicted in the upper panel. The Foxp3 expression (red curve) compared to the isotype (blue curve), and an example of the difference in Foxp3 expression between COPD (red curve) and healthy (blue curve) are depicted in the lower panel.

COPD versus healthy

COPD patients had higher percentages of $CD4^+CD25^+Foxp3^+$T cells (p=0.04, Figure 2A) and $CD4^+CD25^{high}$ $Foxp3^+$T cells (p=0.05, Figure 2C) than healthy individuals. When analyzing the groups based on their current smoking status, COPD smokers had a higher percentage of $CD4^+CD25^{high}Foxp3^+$T cells than healthy smokers (p=0.049, Figure 2D), which was also true for the $CD4^+CD25^+Foxp3^+$T cells (trend (p=0.065), Figure 2C). No differences were found between COPD and healthy individuals with respect to CD4 T cells, $CD4^+CD25^+$ T cells, and $CD4^+CD25^{high}$ T cells (Figure 3).

The differences in percentages of $CD4^+CD25^+Foxp3^+$T cells could not be explained by the difference in age or packyears of smoking between COPD patients and healthy individuals (p> 0.05, when age or packyears was added to the multiple regression analysis).

HO-1 expression was analyzed in $CD4^+CD25^+Foxp3^+$T cells as a crude measure for Treg functionality [11]. Because all cells were positive for HO-1, the results are expressed as mean fluorescence intensity (MFI). There were no significant differences in HO-1 expression of $CD4^+CD25^+Foxp3^+$T cells between COPD patients and healthy individuals (Figure 4).

Effect of current smoking

There were no significant interactions between COPD and current smoking status, nor were there effects of current smoking with respect to percentages of $CD4^+CD25^+Foxp3^+$T cells in peripheral blood or HO-1 expression in these cells.

B cells and memory B cells in peripheral blood

Based on the expression of CD20, CD27, and membrane IgM, different B-cell subsets were distinguished. Within the lymphocyte gate, total B cells were analyzed based on CD20 expression, and total memory B cells were analyzed based on co-expression of CD20 and CD27 (Figure 5). Within the CD20 population, naive B cells ($CD27^-IgM^+$), IgM^+ memory B cells ($CD27^+IgM^+$), and class-switched memory B cells ($CD27^+IgM^-$) were distinguished (Figure 5).

COPD versus healthy

COPD patients had lower percentages of total B cells (p=0.02, Figure 6A) and there was a trend for a lower percentage of memory B cells (p=0.10) compared to healthy individuals. When analyzing the groups based on their current smoking status, there was a lower B cell percentage in COPD ex-smokers compared to healthy smokers (p=0.01), ex-smokers (p=0.02) and never smokers (p=0.03) and a trend (p=0.05) when compared to COPD smokers (Figure 6B).

The lower percentages of B cells in COPD could not be explained by the difference in age or packyears between COPD patients and healthy individuals (p>0.05, when age or packyears was added to the multiple regression analysis).

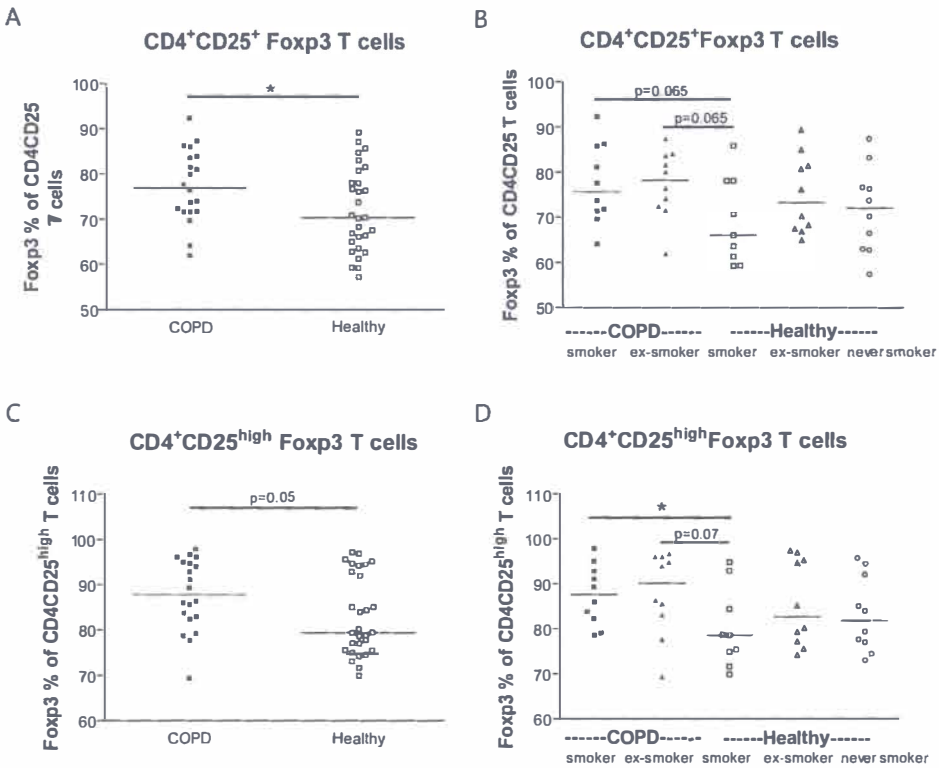


Figure 2. Regulatory T cells in peripheral blood

*A) Foxp3 percentages of CD4+CD25+ T cells and C) CD4+CD25high T cells in peripheral blood of COPD patients (closed symbols) and healthy individuals (open symbols). In B and D the same results are depicted, but divided in subgroups based on the current smoking status. The results of the Mann Whitney U tests are depicted in the figures. * indicates that p< 0.05*

Effect of current smoking

There were no significant interactions between COPD and current smoking status with respect to B cells and memory B cells. However, current smokers (COPD and healthy combined) had higher percentages of memory B cells compared to ex-smokers and never smokers (p<0.01, Figure 7A). When analyzing the effect of current smoking within COPD, COPD smokers had higher percentages of memory B cells than COPD ex-smokers (p= 0.03, Figure 7B). Also within healthy individuals, current smokers had higher percentages of memory B cells than ex-smokers (p=0.03, Figure 7B) and never smokers (p=0.02, Figure 7B). This memory B cell- population in current smokers mainly consisted of class-switched memory B cells, which are characterized by the lack of IgM expression (Figure 7C, p<0.01). When analyzing the groups based on their current smoking status, healthy smokers had higher percentages of class-switched memory B cells than healthy ex-smokers (p<0.01, Figure 7D) and never smokers (p<0.01, Figure 7D).

There were no effects of COPD or current smoking on IgM+ memory B cells and naive B cells (data not shown).

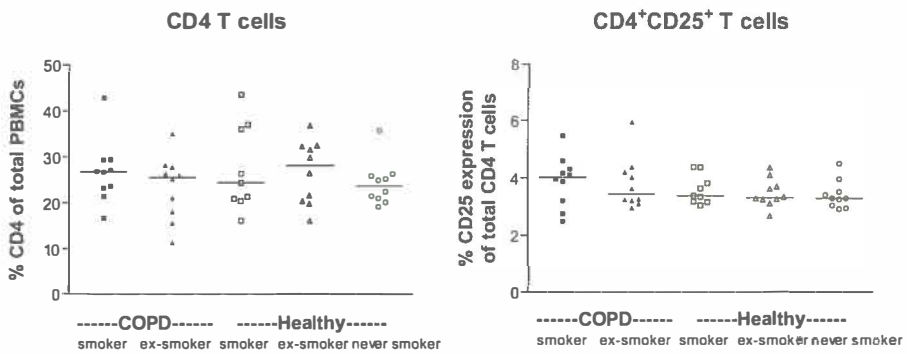
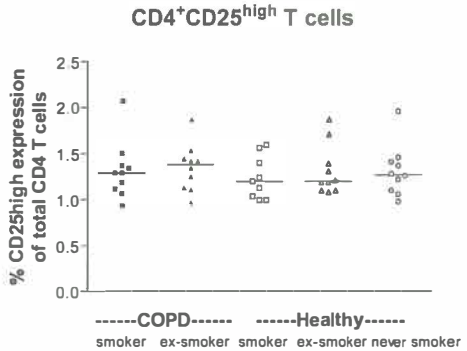


Figure 3: CD4 T cell subsets in peripheral blood

Percentages of CD4+ T cells, CD4+CD25+ T cells and CD4+CD25high T cells in peripheral blood of COPD patients (closed symbols) and healthy individuals (open symbols). There were no significant effects of COPD or current smoking status.



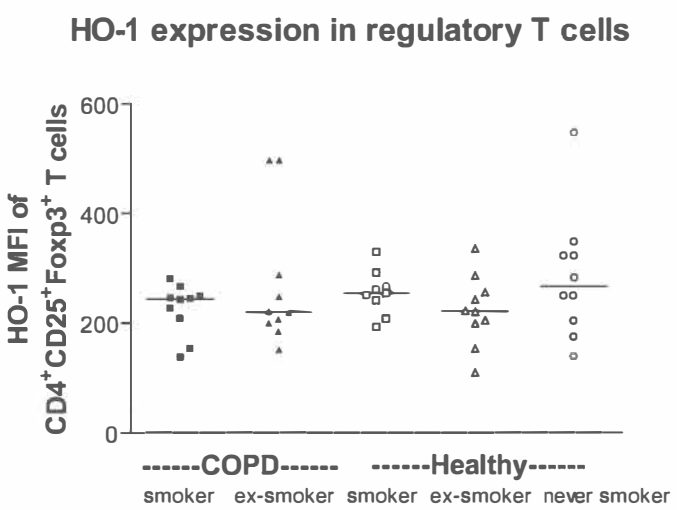


Figure 4: HO-1 expression in regulatory T cells

HO-1 expression in CD4+CD25+Foxp3+ T cells in peripheral blood of COPD patients (closed symbols) and healthy individuals (open symbols). There were no significant effects of COPD or current smoking status.

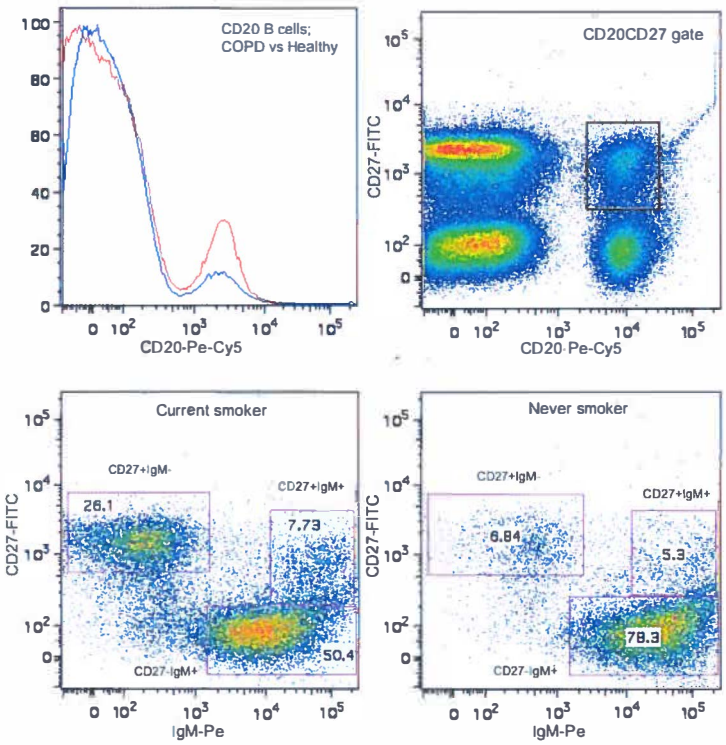


Figure 5. Flow cytometry plots of B cells and memory B cells in peripheral blood. *A representative example of the difference in percentage of CD20⁺ B cells between COPD (blue) and healthy (red) and the CD20⁺CD27⁺ gate to analyze the memory B cells is depicted in the upper panel. The CD27⁺IgM⁻ gate for class switched memory B cells, the CD27⁺IgM⁺ gate for IgM⁺ memory B cells, and the CD27⁻IgM⁺ gate for naïve B cells are shown for a current and a never smoker in the lower panel.*

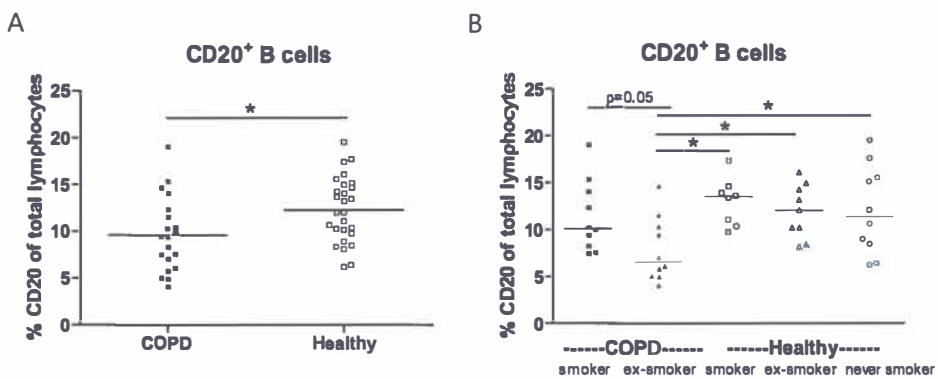


Figure 6: B cells in peripheral blood

*A) Percentages of total B cells, in peripheral blood of COPD patients (closed symbols) and healthy individuals (open symbols). In B the same results are depicted, but divided in subgroups based on the current smoking status. The results of the Mann Whitney U tests are depicted in the figures. * indicates that p< 0.05*

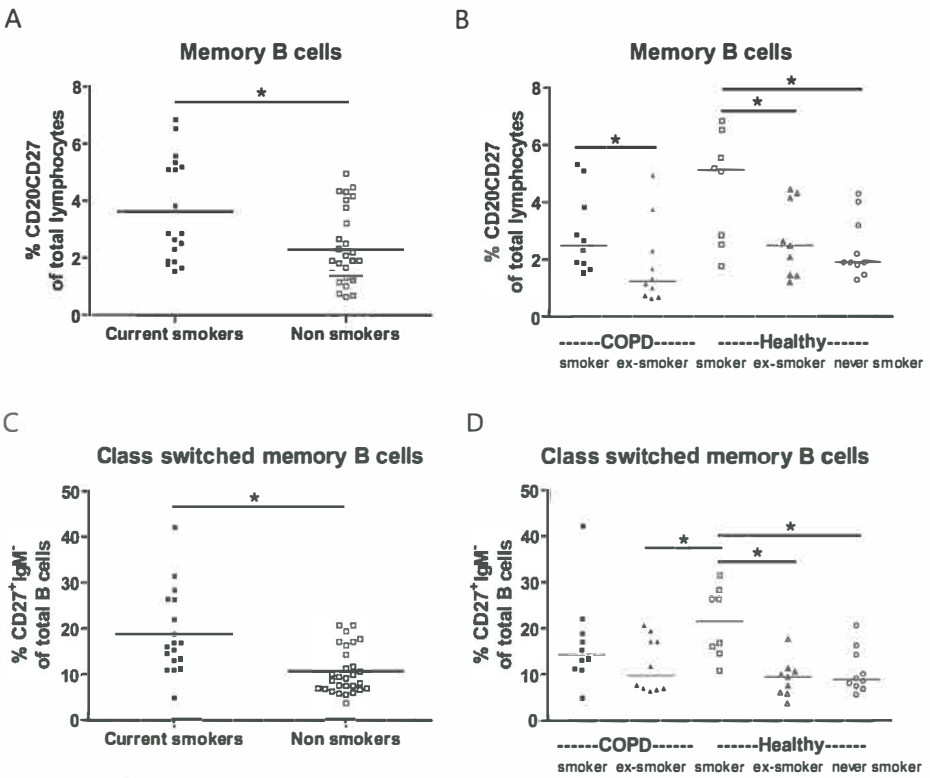


Figure 7: Memory B cells in peripheral blood

*A) Percentages of memory B cells, and class switched memory B cells (C) in peripheral blood of current smokers (closed symbols) and non smokers (open symbols). In B and D the same results are depicted, but divided in subgroups based on the presence of COPD and the current smoking status. The results of the Mann Whitney U tests are depicted in the figures. * indicates that p< 0.05*

Correlation between regulatory T cells and B cells

The percentages of $CD4^+CD25^+Foxp^+$T cells were negatively correlated with the percentage of B cells (Spearman's rho= -0.36, p=0.01, Figure 8) and memory B cells (Spearman's rho= -0.34, p=0.02). For COPD alone, the correlation between $CD4^+CD25^+Foxp^+$T cells and B cells was of the same magnitude, but due to less power it did not reach statistical significance (Spearman's rho = -0.4, p=0.08).

Discussion

We found higher percentages of Tregs and lower percentages of B cells in peripheral blood of patients with COPD compared to healthy individuals. Additionally, higher Treg percentages correlated with lower B cell and memory B cell percentages. Interestingly, higher percentages of memory B cells, particularly class-switched memory B cells, were present in peripheral blood of current smokers, regardless of the disease state.

In addition to our earlier studies regarding B cells in lung tissue of COPD patients [3,6], we now have studied the presence of B cells and memory B cells in peripheral blood of COPD patients and healthy individuals. Except for one earlier publication from our group which showed decreased B-cell percentages in COPD non-smokers compared to COPD smokers [17], we could not find any data assessing the presence of these B-cell populations in peripheral blood of patients with COPD. The current study showed lower percentages of B cells and a similar trend for memory B cells in COPD patients compared to healthy individuals. The lowest B-cell percentages were detected in the COPD ex-smokers, and this is consistent with the earlier findings of de Jong et al. [17]. Although speculative, this decrease in total B cells in peripheral blood in COPD patients and the already described increased presence of B cells in lung tissue of COPD patients [5,6] could reflect an increased recruitment of B cells from the periphery to the lung, probably in response to increased presence of antigens in the lungs. Since B cells were expressed as the percentage of total lymphocytes, the decreased percentage of B cells in peripheral blood in COPD patients could also be due to an increase in T cells, which has already been demonstrated for CD8 T cells in COPD [18].

Intriguingly, there was a strong increase in memory B cells, and in particular class-switched memory B cells in current smokers compared to ex- and never smokers. Class-switched memory B cells are mature B cells that have replaced their primary encoded membrane receptor (IgM) by IgG, IgA or IgE in response to repeated antigen recognition [19]. This process of class-switch recombination is dependent on presence of specific antigen-antibody complexes in germinal centers (GC), and thus the extent of this GC mediated level of class-switching is related to actual presence of antigen and recognizing antibody. The finding of increased class-switched memory B cells in current smokers suggests the possibility of a chronic antigen specific immune response that is particularly caused by ongoing smoke-induced formation or release of (neo)-antigens (e.g. matrix degradation products or smoke particles). The primary immune response to these antigens may be weak, but may still lead to the formation of memory B cells. When the antigen stimulus (tobacco smoke) is present for a prolonged period, secondary immune responses may lead to increased numbers of memory B cells and plasma cells, and a continued presence of memory B cells, as shown in the current smokers in our study.

As mentioned in the introduction, the presence of $CD4^+CD25^+$ Tregs in COPD has been investigated in three earlier studies. These studies reported different findings in lung tissue and bronchoalveolar lavage (BAL), and found no differences in $CD4^+CD25^+$ Tregs in peripheral blood. Two studies investigated Tregs in BAL; the first showed increased numbers of $CD4^+CD25^{bright}$ Tregs in COPD patients and healthy smokers compared to healthy never smokers [15], while the second study showed decreased $CD4^+CD25^+$ Tregs in COPD patients and never smokers compared to healthy smokers [16]. These differences in the BAL results are remarkable, and may be explained by the different gating of the CD25 expression; i.e. $CD25^+$ versus $CD25^{bright}$. In addition, the current smokers of the second study had to refrain from smoking 12 hours before bronchoscopy to avoid acute smoke effects, whereas this was not mentioned in the other study. This could also explain different findings in BAL. In contrast to our study, none of these studies found

differences in Tregs in peripheral blood of COPD patients and healthy individuals. Similar to the other studies we also assessed the presence of $CD4^+CD25^+$ T cells in peripheral blood of COPD patients and healthy individuals, and detected no differences in $CD4^+CD25^+$ or $CD4^+CD25^{high}$ T cells between COPD patients and healthy individuals, nor were effects of current smoking observed. However, we analyzed Tregs by measuring the percentage of Foxp3 expressing $CD4^+CD25^+$ T cells and with this method increased Treg percentages in peripheral blood of COPD patients were found when compared to healthy individuals. The findings of Lee et al [14] showing a decreased presence of Tregs in the lung in COPD patients together with our observed increase in peripheral blood could suggest a decreased infiltration of Tregs to the lung in COPD.

Notwithstanding the relevance of these findings, the functionality of Tregs is probably more important than the mere presence of these cells. Lee et al were the first to investigate Treg function in COPD, and found similar Treg function in peripheral blood of COPD patients and healthy individuals [14]. Based on earlier findings [11], HO-1 expression was used as a crude measure for Treg functionality in our study. Although this is a surrogate marker for Treg functionality and not comparable to the standard suppression assays, we also did not find differences in Treg function between COPD patients and healthy individuals with this method, nor effects of current smoking. However, since Treg function can be different at the site of inflammation, depending on the local inflammatory environment [20], overall conclusions as to their relevance in COPD can only be drawn when investigating local Tregs in the lung.

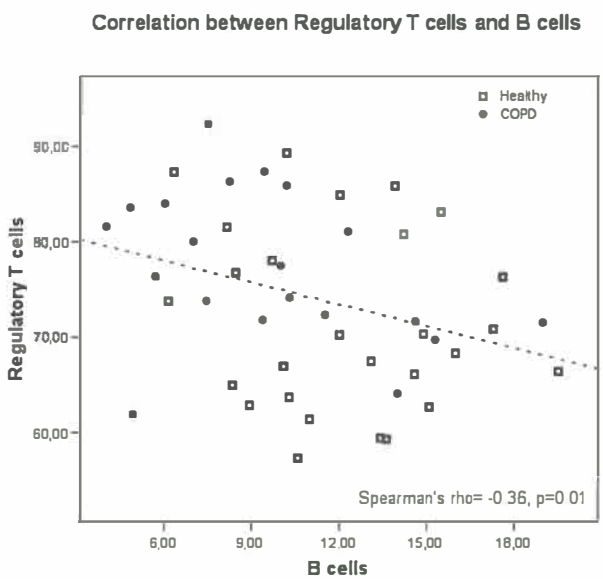


Figure 8: Correlation between regulatory T cells and B cells
Correlation between $CD4^+CD25^+Foxp3^+$T cells and total B cells for COPD patients (black circles) and healthy individuals (open squares). The result of the Spearman correlation is depicted in the figure.

The presence of Tregs and B cells was only assessed in peripheral blood in this study; this does not necessarily reflect the inflammatory response in the lungs. Indeed, our findings confirmed this and as already mentioned, it is appropriate to study presence and particularly functionality of local Tregs in the lung. However, when regarding B cells, this study showed for the first time an increased percentage of (class switched) memory B cells in peripheral blood of current smokers. This finding shows that smoking, which primarily has its effects in the lungs, can also have systemic effects leading to increased levels of circulating memory B cells. Given the fact that B cells traffic to the circulation after antigen recognition in the lung, this smoke induced response might be of a different magnitude when analyzing the lung compartment.

Combining our findings with those in the existing literature, we propose that smoking elicits an antigen specific immune response in addition to the effects on the innate immune system. This antigen specific immune response is possibly directed against lung tissue matrix degradation products or smoke particles. In healthy smokers this smoke-induced immune response is still counterbalanced by a normally functioning specific immune response, including adequate Treg function. In COPD patients, there is an altered specific immune response with increased percentages of Tregs and decreased percentages of B cells in peripheral blood together with a decrease of Tregs and an increase of B cells in the lungs [5,6,14]. This altered specific immune response in COPD may contribute to the development and/or persistence of the chronic inflammatory response in the lungs of patients with COPD.

Our study supports the hypothesis that specific immunity is important in COPD. Future studies focusing on the specific immune response in COPD should further elicit the functionality of local Tregs in the lung, including the influence of the local environment on these cells, and the antigen(s) causing the presumed smoke-induced memory B cell response.

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Chapter 6

Heme oxygenase-1 prevents smoke induced B-cell infiltrates: *a role for regulatory T cells*?

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Abstract

Smoking is the most important cause for the development of COPD. Since not all smokers develop COPD, it is obvious that other factors must be involved in disease development. We hypothesize that heme oxygenase-1 (HO-1), a protective enzyme against oxidative stress and inflammation, is insufficiently upregulated in COPD.

The effects of HO-1 modulation on cigarette smoke induced inflammation and emphysema were tested in a smoking mouse model.

Mice were either exposed or sham exposed to cigarette smoke exposure for 20 weeks. Cobalt protoporphyrin or tin protoporphyrin was injected during this period to induce or inhibit HO-1 activity, respectively. Afterwards, emphysema development, levels of inflammatory cells and cytokines, and the presence of B-cell infiltrates in lung tissue were analyzed.

Smoke exposure induced emphysema and increased the numbers of inflammatory cells and numbers of B-cell infiltrates, as well as the levels of inflammatory cytokines in lung tissue. HO-1 modulation had no effects on smoke induced emphysema development, or the increases in neutrophils and macrophages and inflammatory cytokines. Interestingly, HO-1 induction prevented the development of smoke induced B-cell infiltrates and increased the levels of $CD4^+CD25^+$ T cells and Foxp3 positive cells in the lungs. Additionally, the $CD4^+CD25^+$ T cells correlated positively with the number of Foxp3 positive cells in lung tissue, indicating that these cells were regulatory T cells.

These results support the concept that HO-1 expression influences regulatory T cells and indicates that this mechanism is involved in the suppression of smoke induced B-cell infiltrates. The translation of this interaction to human COPD should now be pursued.

Background

Chronic obstructive pulmonary disease (COPD) is a major global health problem with increasing morbidity and mortality. Smoking is widely accepted as the most important cause for development of the disease, still 'only' 15-20% of the smoking population eventually develops COPD [1]. COPD is characterized by a chronic inflammatory process, which ultimately leads to airway obstruction and emphysema. The important role of neutrophils, macrophages and cytotoxic T cells in its development is well established [2], yet the role of CD4 T cells and B cells has only recently re-attracted attention. We and others have found oligoclonal T- and B cells in the lungs of COPD patients suggesting an antigen driven immune response [3,4]. These T-and B cells are aggregated in lymphoid infiltrates. Similar infiltrates have been shown in the lungs of mice chronically exposed to cigarette smoke [3]. We hypothesize that these lymphoid infiltrates contribute to the development and/or persistence of the inflammatory response in COPD [3].

Since not all patients with COPD have actively smoked, cigarette smoke cannot be the sole contributing factor in COPD development. Other factors involved are genetic factors, such as α1 anti-trypsin deficiency, and environmental factors, such as air pollution. Another intriguing factor that may play a role in COPD development is the 'protective' enzyme heme oxygenase-1 (HO-1). HO-1 is the rate limiting enzyme involved in the breakdown of heme to equimolar amounts of biliverdin, free iron and carbon monoxide (CO). HO-1 is rapidly upregulated with oxidative stress and has potent anti-inflammatory, anti-apoptotic and anti-proliferative effects [5-7]. The anti-inflammatory and cytoprotective effects of HO-1 are mediated by its products, of which in particular CO [8-10]. Notwithstanding this knowledge, the exact mechanisms behind the protective effects of HO-1 are still poorly understood.

Interestingly, a reduced HO-1 expression in macrophages in lung tissue and bronchoalveolar lavage (BAL) in patients with COPD has been shown [11,12]. In some people this may be due to a genetic polymorphism in the HO-1 promoter gene, which causes a lower HO-1 inducibility by reactive oxygen species (ROS) [13]. Additionally, adenoviral mediated HO-1 overexpression in the lung suppresses porcine pancreatic elastase induced emphysema development in mice [14], again suggesting involvement of HO-1 in emphysema development.

Our general hypothesis is that if HO-1 is insufficiently upregulated, this contributes to a higher susceptibility to noxious effects of cigarette smoke and subsequent development of COPD. We tested whether HO-1 modulation in our smoking mouse model [3] influences the development of cigarette smoke induced emphysema and lung inflammation, in particular with respect to lymphoid infiltrates. We hypothesized that HO-1 induction attenuates cigarette smoke induced emphysema and inflammation and conversely HO-1 inhibition worsens the noxious effects of cigarette smoke.

This study showed that long term HO-1 upregulation prevented the development of cigarette smoke induced B-cell infiltrates, while it had no effect on smoke induced emphysema and increase in inflammatory cells and cytokines. Increased

numbers of $CD4^+CD25^+$ Tregs could be an explanation for the reduced presence of these B-cell infiltrates.

Methods

Study design

Female A/J mice were divided into six groups (n=11 per group); 1. Phosphate buffered saline (PBS) + smoke, 2. Cobalt protoporphyrin (CoPP) + smoke, 3. Tin protoporphyrin (SnPP) + smoke, 4. PBS + sham smoke, 5. CoPP + sham smoke, 6. SnPP + sham smoke. During 20 weeks the mice were subjected to protoporphyrin (or PBS) treatment and smoke (or sham smoke) exposure. After 20 weeks the mice were sacrificed, the trachea was cannulated, the right lung was ligated, and lung lobes were either snap-frozen and stored at -80°C (n=7) or freshly used for flow cytometry analysis (n=7). The left lung was inflated, and fixed for 24h with formalin with a constant pressure of 25cm H_2O (n=8).
Experiments were approved by the local committee on animal experimentation.

Smoke exposure

Mice were exposed to 24 puffs of cigarette smoke from two 2R1 reference cigarettes (University of Kentucky) two times per day, for 5 days a week during 20 weeks, as described previously [3].

Protoporphyrin treatment

CoPP and SnPP (Frontier Scientific, Logan, USA) were dissolved in 1M NaOH, diluted to the proper concentration with PBS and adjusted to pH 7.3-7.5 with HCl. The mice received a subcutaneous injection with CoPP (25μM/kg = 16.4mg/kg) every two weeks, or with SnPP (10μM/kg = 7.5mg/kg), or PBS weekly. These concentrations and dosing regimens were based on a pilot, in which different protoporphyrin concentrations were tested for a maximum period of two weeks.

Morphometrical evaluation of emphysema

Alveolar airspace enlargement was assessed by mean linear intercept (Lmi) by two independent individuals in a blinded manner, as described previously [3,15].

Cytokines

Frozen lung tissue was homogenized in 50 mM Tris-HCl buffer, containing 150 mM NaCl, and 0.002% Tween-20 (pH 7.5) and centrifuged at 12000 g for 10 min to remove any insoluble material. Concentrations of TNF-α, IL-1α, IL-1β, IL-6, KC (mouse IL-8) and MCP-1 (monocyte chemoattractant protein-1) in supernatants were measured with a multiplex ELISA system (Lincoplex Systems, St Charles, MO, USA).

Flow cytometry

Single-cell leukocyte suspensions were obtained from lungs for flow cytometric analysis as described previously [16]. Numbers of $CD4^+CD25^+$ T cells and neutrophils were calculated based on the label combinations: CD3-APC, CD4-PE,

CD25-FITC and Gr1-APC. All antibodies were obtained from Pharmingen (San Diego, USA).

Histology

HO-1 expression was demonstrated with the rabbit polyclonal antibody anti-HO-1 (Stressgen, Victoria, Canada). Macrophage numbers were identified with an anti-Mac3 antibody (Pharmingen) and were quantified by morphometric analysis using Leica Qwin image analysis software (Leica Microsystems BV, Rijswijk, the Netherlands). With this computerized method the total Mac3 positive stained surface area was measured and divided by the total surface area lung tissue, and expressed as volume percentages [16]. B-cell infiltrates were detected with an anti-B220 antibody (Pharmingen). The total surface of the B220 positive infiltrates (clusters of at least 10 cells) was quantified by morphometric analysis and divided by the total surface area lung tissue, and expressed as volume percentages. Forkhead transcription factor 3 (Foxp3) expression, a marker for regulatory T cells, was detected in 4 µm sections of frozen lung tissue by staining with a monoclonal anti-Foxp3 antibody (Alexis, Breda, the Netherlands). The total number of Foxp3 positive cells was counted at 25x magnification and expressed per surface area lung tissue determined by morphometric analysis. A fluorescent double staining with hamster anti mouse-CD3 (Pharmingen), followed by mouse anti hamster FITC-labeled (eBioscience) and rat anti mouse-Foxp3 (Alexis) followed by biotin conjugated goat anti rat (SBA, Birmingen, USA) and Strep-APC (Pharmingen) was performed on 4 µm frozen sections of spleen and lung tissue to confirm that Foxp3 positive cells were T cells.

Western blot analysis

HO-1 protein expression was measured with western blot in whole lung homogenate (see cytokine analysis). The proteins were separated for molecular weight and blotted on a nitrocellulose membrane. The membrane was blocked overnight in 5% skim milk and incubated with rabbit-anti-HO-1 (Stressgen) followed by a peroxidase labeled goat-anti-rabbit antibody (DakoCytomation, Heverlee, Belgium). For protein loading control the membrane was stripped using a 25mM Glycine-HCl buffer containing 1% SDS (pH:2) and stained for β-actin (loading control, Abcam, Cambridge, UK) followed by a peroxidase labeled goat-anti-rabbit antibody (DakoCytomation). The bands of interest were visualized using enhanced chemiluminescence according to standard methods.

Statistics

A multiple linear regression model was used to establish importance of smoke exposure and protoporphyrin treatment and their possible interactions [17]. First, the model was tested with the effects of smoking, CoPP treatment, SnPP treatment, together with the interactions between smoking and CoPP, and smoking and SnPP. When the interactions were not significant the model was tested again without the interaction terms. Afterwards the normal distribution of the residuals was analyzed and when needed the data were log or 1/x-transformed to normalize distributions. A significant interaction signifies that the effect of the combination is different (larger or smaller) than the addition of the separate effects of the exposures.

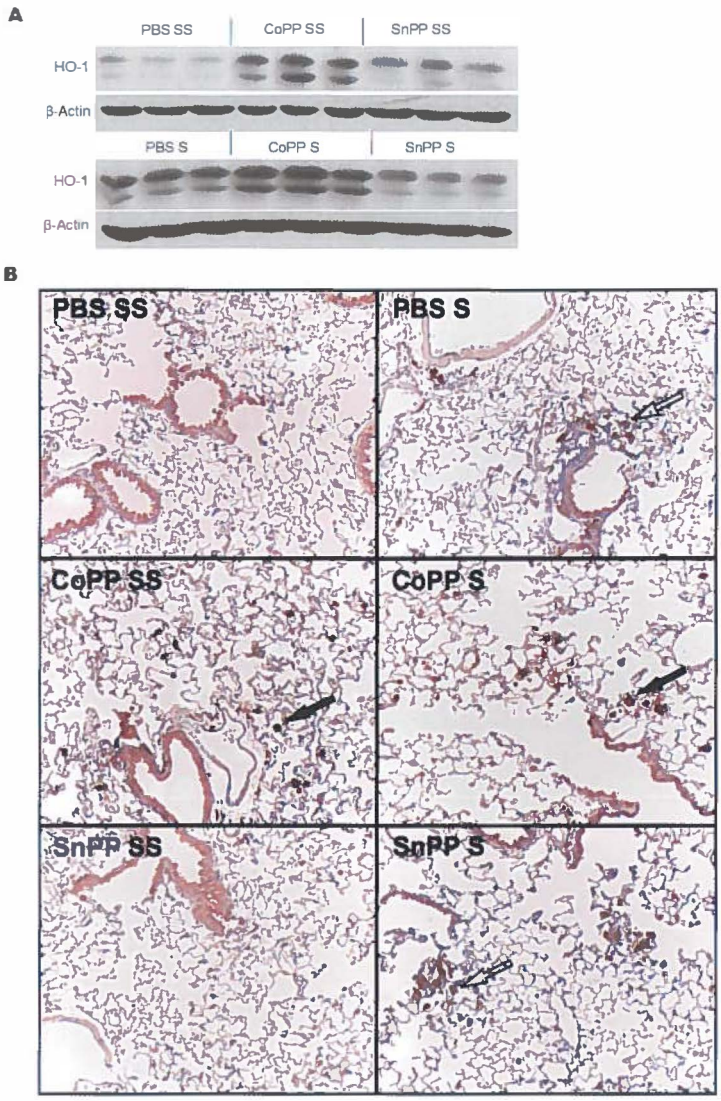


Figure 1: HO-1 protein expression.

A: Protein bands for HO-1 (above band) and β-actin (loading control) detected by western blot analysis after long term smoke exposure and protoporphyrin treatment. Three animals per group are shown.

B: A representative picture of the HO-1 expression (dark red) in lung tissue is shown for each group (25x). Particularly, alveolar macrophages (indicated with a closed arrow) show an increased HO-1 expression after CoPP treatment. The epithelium stains faintly in all groups and no differences were observed between the groups. The brown cells (indicated with an open arrow) are pigmented macrophages, a result of the smoke exposure. S: smoke, SS: Sham smoke.

Mice were divided into 6 groups; 1. Phosphate buffered saline (PBS) + smoke, 2. Cobalt protoporphyrin (CoPP) + smoke, 3. Tin protoporphyrin (SnPP) + smoke, 4. PBS + sham smoke, 5. CoPP + sham smoke, 6. SnPP + sham smoke.

Mann Whitney U tests were used for post-hoc analysis to test whether significant effects of CoPP and SnPP treatment were present only in smokers or sham smokers or in both groups. $CD4^+CD25^+$ T cells and Foxp3 positive cells were evaluated with the Spearman correlation. A value of $p<0.05$ was considered significant.

Results

Protoporphyrin treatment and smoking upregulate HO-1 expression

CoPP resulted in a clear upregulation of HO-1 protein expression in the lung, particularly in alveolar macrophages (Figure 1). Smoking also resulted in an increased HO-1 protein expression, leading to highest levels of HO-1 in smoke-exposed mice that also received CoPP. SnPP resulted in a small increase in HO-1 expression, which was not affected by smoking.

No effects of HO-1 modulation on smoke induced emphysema development

Smoking induced emphysema after 5.5 months smoke exposure, expressed as a significant increase in mean linear intercept (Figure 2, $p< 0.01$). There were no effects of both protoporphyrins on the mean linear intercept.

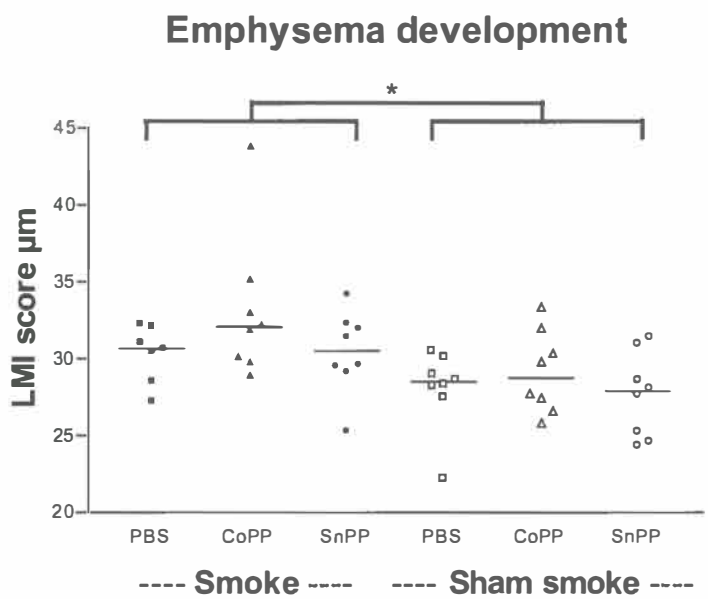


Figure 2: Emphysema development.
*Mean linear intercept (LMI) after long term smoke exposure and protoporphyrin treatment. Smoke groups are represented by closed symbols and sham smoke groups by open symbols. * indicates a significant effect of smoke exposure ($p<0.05$). There were no interactions and no effects of CoPP or SnPP treatment.*

Smoking increases the levels of inflammatory cytokines in lung tissue

Smoking significantly increased the levels of the pro inflammatory cytokines TNF-α, IL-1α, IL-1β, IL-6, KC, and MCP-1 in lung homogenate (Figure 3, $p<0.01$). CoPP increased the levels of IL-6 and KC. Levels of IL-6 were significantly increased after CoPP only in the sham smokers (PBS sham smoke vs. CoPP sham smoke $p<0.05$). KC levels were increased after CoPP in both smokers and sham smokers (PBS sham smoke vs. CoPP sham smoke and PBS smoke vs. CoPP smoke $p<0.01$). In contrast, SnPP reduced the levels of TNF-α, IL-1α, IL-1β, KC and MCP-1.
Levels of these cytokines were significantly decreased after SnPP in the sham smokers only (PBS sham smoke vs. SnPP sham smoke $p<0.05$). Additionally, there was a positive interaction between SnPP and smoking for KC ($p<0.01$) leading to higher KC levels in the SnPP smokers compared to the SnPP sham smokers.

Smoking increases neutrophils and macrophages in lung tissue

Smoking increased the numbers of neutrophils and macrophages in the lung (Figure 4, $p<0.01$). There were no effects of both protoporphyrins on the numbers of neutrophils and macrophages.

CoPP treatment prevents cigarette smoke induced B-cell infiltrates

Smoking significantly increased the number of B-cell infiltrates in lung tissue (Figure 5, $p<0.01$). In addition, there was a significant negative interaction between smoking and CoPP ($p<0.01$) signifying that the smoke induced increase in B-cell infiltrates was reduced in the smoke-exposed mice that also received CoPP (CoPP smokers).

Reduced number of B-cell infiltrates in CoPP smokers is accompanied by increased numbers of $CD4^+CD25^+$ T cells in lung homogenate

Smoking significantly increased the numbers of $CD4^+CD25^+$ T cells in lung homogenate (Figure 6A, $p<0.01$). Additionally, there was a significant positive interaction between smoking and CoPP treatment for the numbers of $CD4^+CD25^+$ T cells ($p<0.05$), signifying that the increase of smoking was larger in combination with CoPP, resulting in the highest numbers of $CD4^+CD25^+$ T cells in the CoPP smokers. This higher expression of CD25 in the $CD4^+$ T-cell population of CoPP smoking compared to PBS smoking mice is illustrated in figure 6B.

The increase in $CD4^+CD25^+$ T cells represents an increase in regulatory T cells

To investigate whether the increased number of $CD4^+CD25^+$ T cells in the CoPP smokers represented an increase in regulatory T cells (Tregs), we stained lung tissue for the Treg specific marker Foxp3 (Figure 7A). Smoking significantly increased the numbers of Foxp3 positive cells in lung tissue (Figure 7B, $p<0.01$) with a trend ($p=0.07$) for a similar effect of CoPP on the number of Foxp3 positive cells. Double staining for CD3 and Foxp3 in lung and spleen tissue (Figure 7C) showed that Foxp3 positive cells were indeed T cells. Furthermore, the number of Foxp3 positive cells in lung tissue correlated positively with the number of $CD4^+CD25^+$ T cells in lung homogenate ($\rho = 0.7$, $p<0.01$).

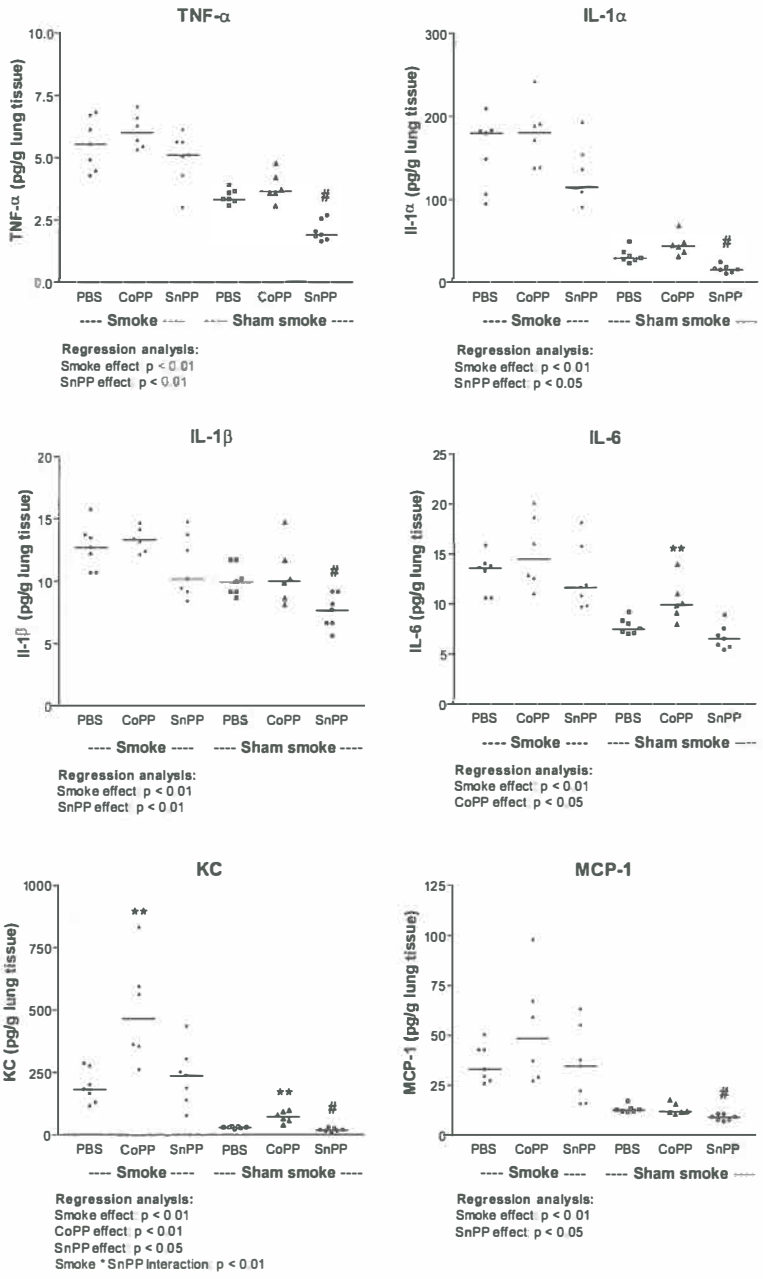


Figure 3: Inflammatory cytokines in lung homogenate

*TNF-α, IL-1α, IL-1β, IL-6, KC and MCP-1 levels expressed as pg/g lung homogenate after long term smoke exposure and protoporphyrin treatment. Smoke groups are represented by closed symbols and sham smoke groups by open symbols. The significant results of the regression analysis are depicted beneath the figures. # indicates a significant effect of SnPP treatment (SnPP vs. PBS, post-hoc analysis), and ** indicates a significant effect of CoPP treatment (CoPP vs. PBS, post-hoc analysis) (p<0.05).*

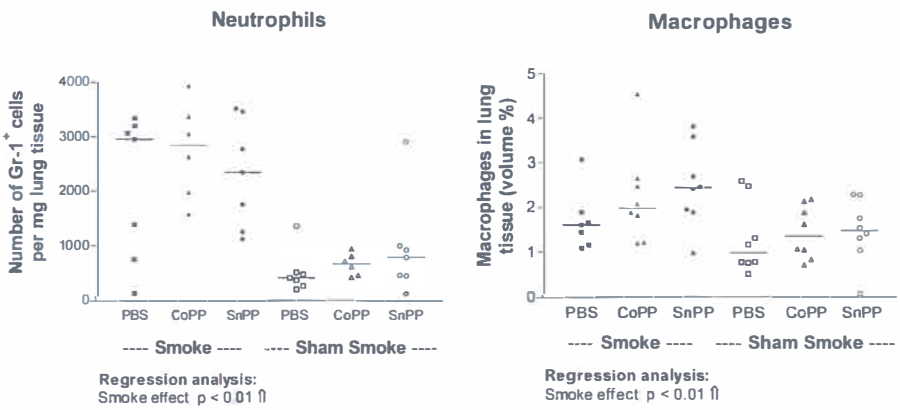


Figure 4: Inflammatory cells in lung tissue

Neutrophils and macrophages expressed in lung tissue after long term smoke exposure and protoporphyrin treatment. Neutrophils are expressed as numbers per mg lung homogenate. Macrophages are expressed as volume percentages. Smoke groups are represented by closed symbols and sham smoke groups by open symbols. The significant results of the regression analysis are depicted beneath the figures.

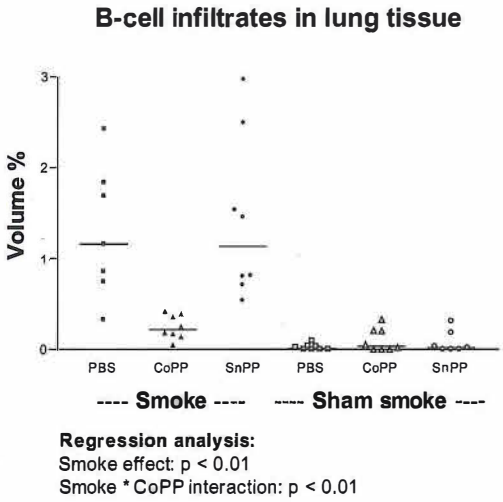
Discussion

In this study we showed that HO-1 protein upregulation by CoPP treatment reduced the number of cigarette smoke induced B-cell infiltrates in mice. These B-cell infiltrates were similar to the lymphoid follicles found in COPD patients [3] and are suggested to contribute to COPD development. The reduced number of B-cell infiltrates in the CoPP smokers was accompanied by increased numbers of $CD4^+CD25^+$T cells, which most likely are Tregs. In contrast to our hypothesis, HO-1 upregulation had no protective effect on cigarette smoke induced increases in other inflammatory cells and inflammatory cytokines and subsequent emphysema development. Additionally, SnPP treatment did not aggravate smoke induced damage.

We succeeded in long term HO-1 protein upregulation in our smoking mouse model and to our knowledge this is the first study using an intervention that leads to long term HO-1 protein upregulation in vivo. Given the importance of macrophages and epithelium in the production of inflammatory mediators after an inflammatory or oxidative stimulus, these cells were carefully evaluated for their HO-1 expression. In both in vitro and in vivo studies oxidative stress and cigarette smoke have been shown to induce HO-1 expression in pulmonary epithelial cells and alveolar macrophages [18-21]. Furthermore, HO-1 overexpression in epithelial cells is protective against oxidative stress [22]. Indeed, our study showed an increased HO-1 expression after both cigarette smoke exposure and CoPP treatment. This increased HO-1 expression was highest in CoPP treated smoking mice and particularly seen in alveolar macrophages yet not in epithelial cells. Unfortunately, HO-1 upregulation provided no protective effects against the

smoke induced increases in inflammatory cells and cytokines, nor did it protect against smoke induced emphysema. These results do not fit with our hypothesis, but might be explained in several ways. Firstly, the epithelium did not show an increased HO-1 expression after CoPP treatment, which still makes it possible for these cells to respond to cigarette smoke by producing inflammatory mediators. In fact, the majority of the inflammatory cytokines that were increased after smoke exposure in our study can be produced by epithelial cells in response to cigarette smoke [23-26], which supports this option.

A



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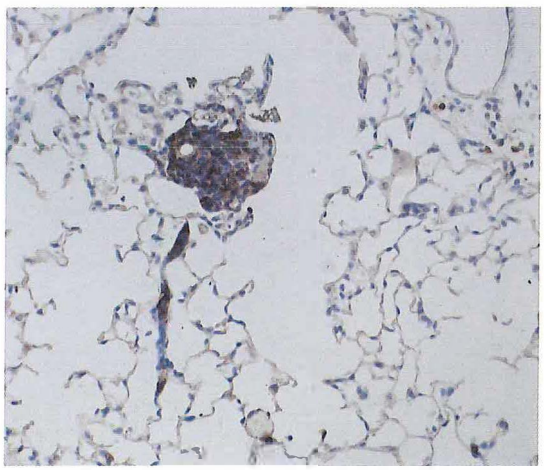
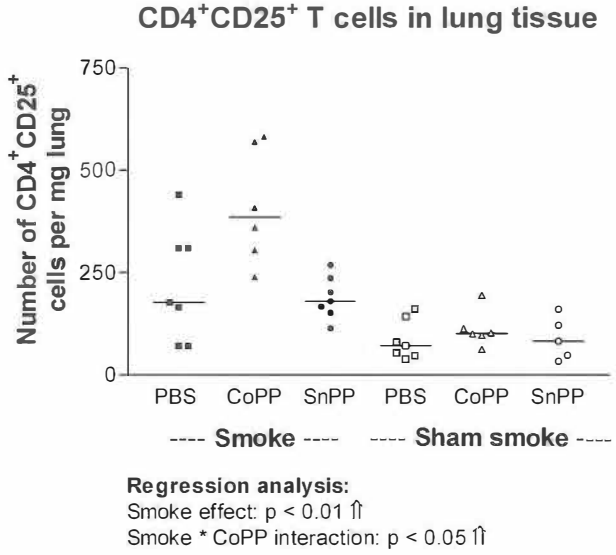


Figure 5: B-cell infiltrates in lung tissue.
A: Volume percentage of B220 positive infiltrates after long term smoke exposure and protoporphyrin treatment. Smoke groups are represented by closed symbols and sham smoke groups by open symbols. The significant results of the regression analysis are depicted beneath the figure.
B: Example of a B-cell infiltrate positive for B220 (red) present in lung tissue (50x).

Secondly, contrary to what we had expected, the levels of IL-6 and KC increased after CoPP treatment in lung tissue, which may suggest some toxicity of the long term dosing of CoPP. Given the fact that the majority of the mice showed irritation of the skin at the injection site after approximately 4 months of CoPP treatment, the CoPP dose indeed might have been too high.

A



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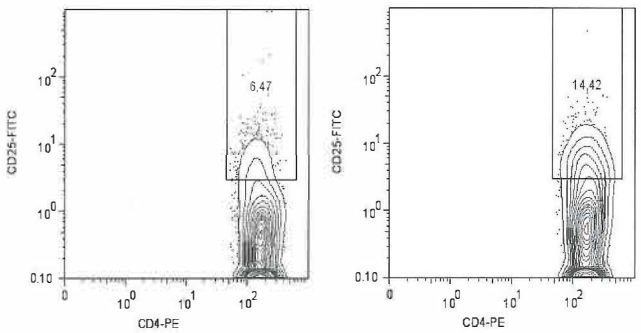

Figure 6: CD4⁺CD25⁺ T cells in lung tissue
A: CD4⁺CD25⁺ T cells expressed as numbers per mg lung tissue after long term smoke exposure and protoporphyrin treatment. Smoke groups are represented by closed symbols and sham smoke groups by open symbols. The significant results of the regression analysis are depicted beneath the figure.
B: Example of the flow cytometry analysis: The percentage of CD25 positive cells within the CD4⁺ T cell population is shown for a PBS (left) and a CoPP (right) smoking mouse.

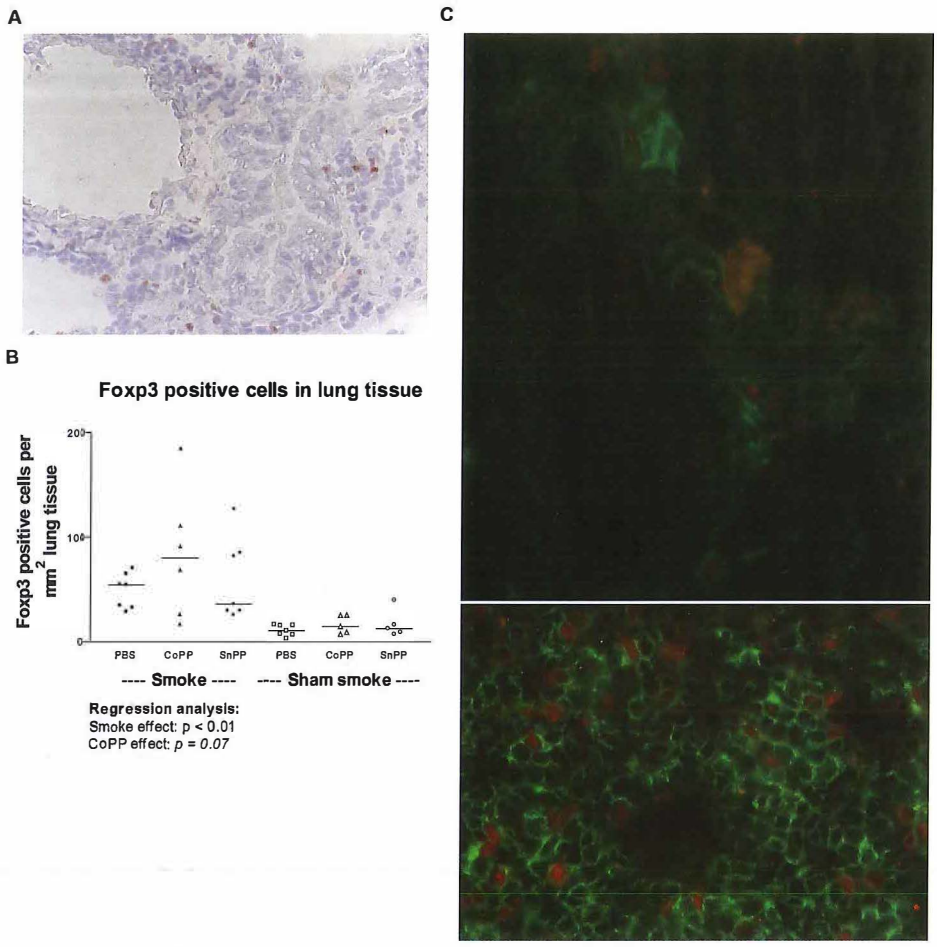

Figure 7: Foxp3 positive cells in lung tissue

A: Foxp3 positive cells (red nuclear staining) present in lung tissue (100x).

B: Foxp3 positive cells expressed as total numbers per mm² lung tissue after long term smoke exposure and protoporphyrin treatment. Smoke groups are represented by closed symbols and sham smoke groups by open symbols. The significant results of the regression analysis are depicted beneath the figure, as well as a trend for an effect of CoPP treatment.

C: Fluorescent double staining for CD3 (green) and Foxp3 (red) in lung (upper panel, 630x) and spleen (lower panel, 400x) showing that Foxp3 specifically stains T cells. The orange cells in the lung are pigmented macrophages, which show auto fluorescence.

It is also conceivable that long term exposure to CoPP, much longer than performed by others, might have other unexpected effects. For future long term experiments it is probably more appropriate to use HO-1 transgenic mice, or use less toxic downstream products of the HO-1 system e.g. CO or bilirubin. Finally, it was not possible to reliably measure HO-activity levels on the frozen material

available in this study; a sufficiently sensitive assessment of HO-activity should certainly be included in future experiments.

SnPP treatment resulted in a slightly increased HO-1 protein expression, which was not affected by smoke exposure. In contrast to our hypothesis, SnPP treatment did not aggravate the damaging effects of smoke exposure, but did decrease the levels of several inflammatory cytokines. SnPP is known to inhibit the HO-1 activity, while it increases HO-1 protein expression [27], which supports the increased HO-1 expression after SnPP treatment in our study. For SnPP treatment both inflammatory and anti-inflammatory effects have been described [28-31]. Anti-inflammatory effects were shown while the HO-1 protein level was increased but the HO-1 activity downregulated, suggesting that HO-1 induction by SnPP can have anti-inflammatory and anti-apoptotic effects independently of the HO-1 enzyme activity [30,31].

The most important finding of this study was the protective effect of HO-1 upregulation on the development of cigarette smoke induced B-cell infiltrates, leading to reduced numbers of B-cell infiltrates in CoPP treated smoking mice. The B-cell infiltrates consisted mainly of B cells surrounded by T cells and were comparable to the B-cell follicles found in patients with COPD [3]. B cells in these follicles were found to be oligoclonal in nature [3], suggesting an antigen driven immune response. Whether the inflammatory response in COPD is a true antigen specific response is not fully proven, nor is it clear which antigen(s) may be involved. We consider matrix degradation products, microbial components, and cigarette smoke constituents as possible candidates. We hypothesize that these lymphoid infiltrates contribute to the development and/or persistence of the inflammatory response in COPD. This study showed that reduced numbers of B-cell infiltrates did not prevent smoke induced emphysema development, which suggests that the presence of B-cell infiltrates may not be a mandatory prerequisite for emphysema development in this model. This is compatible with the results of d'Hulst et al, showing smoke induced emphysema development in scid mice, lacking functional B- and T-cells [32]. To what extent B cells contribute to the persistence of the inflammatory response in COPD remains unclear. However, since these mouse models of cigarette smoke induced emphysema resemble mild disease, it is also possible that B cells might be more important in severe than in mild disease. This would be supported by the data of Hogg et al who found B-cells especially in GOLD stage III and IV [33].

In this study we extended our previous observations on B-cell infiltrates [3] by the intriguing finding that the reduced number of B-cell infiltrates was accompanied by increased numbers of $CD4^+CD25^+$ T cells in the CoPP smokers. The $CD4^+CD25^+$ T-cell population consists of a mixture of activated T cells and Tregs. Tregs are important in controlling immunological tolerance and preventing auto-immune reactions by inhibiting T-cell responses [34,35]. Dysfunction of Tregs can lead to auto-immune diseases, allergy, and chronic inflammatory diseases. The currently best described subset of Tregs is that of the naturally occurring Tregs, expressing high levels of CD25 and the transcription factor Foxp3 [34].

In this study, the number of $CD4^+CD25^+$ T cells correlated positively with the number of Foxp3 positive cells and the highest numbers of Foxp3 positive cells were present in the CoPP smokers with a trend for an effect of CoPP treatment.

Together, this suggests that the increase in $CD4^+CD25^+$ T cells in the CoPP smokers represents an increase in Tregs.
Interestingly, a direct link between Foxp3 and HO-1 expression and function of Tregs was reported recently; both Foxp3 and HO-1 were shown to be expressed in Tregs and the suppressive effects of Tregs were shown to be mediated by HO-1 expression [36]. Furthermore, in a model of allergic airway inflammation, HO-1 upregulation was shown to increase Treg numbers and their suppressive capacity [37].
Next to their effects on T cells, Tregs can also directly suppress B-cell responses without having to suppress the adjacent T cells [38,39]. This proves that activated T cells are not the only target for Tregs and that Tregs can also be involved in the reduced presence of B-cell infiltrates. Interestingly, chronic cigarette smoke exposure was shown to increase the numbers of Tregs in the airways of healthy smokers and smokers with COPD [40], whereas decreased Treg numbers were found in lung tissue of emphysema patients [41]. Additionally, we found high numbers of Foxp3 positive cells present in and surrounding B cell follicles in the lungs of COPD patients (unpublished results).
Altogether, these findings suggest a role for Tregs in COPD in the smoke induced inflammatory response, and possibly also B-cell follicle formation, and support the idea that the HO-1 protein upregulation affected the Treg population in our model thereby possibly contributing to the observed reduced presence of B-cell infiltrates.

Conclusions

Long term HO-1 upregulation prevented the development of cigarette smoke induced B-cell infiltrates, while it had no effect on smoke induced emphysema and increase in neutrophils and macrophages and inflammatory cytokines. A possible explanation for this effect of HO-1 upregulation on presence of B-cell infiltrates is the increased presence of $CD4^+CD25^+$ Tregs. The exact role of these Tregs in the smoke induced inflammatory response has to be elucidated and the translation to human COPD should now be pursued.

Acknowledgements

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6

Chapter 7

Dry powder inhalation of hemin to induce heme oxygenase expression in the lung

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Abstract

The purpose of this study was to formulate hemin as a powder for inhalation and to show proof of concept of heme oxygenase 1 (HO-1) expression in the lungs of mice by inhalation of hemin. Hemin was spray dried from a neutralized sodium hydroxide solution. The particle size distribution of the powder was between 1-5 μm. Dispersion from the Twincer dry powder inhaler showed a fine particle fraction (<5 μm) of 36%. A specially designed aerosol box based on the Twincer®-inhaler was used for a proof of concept study of HO-1 induction by inhalation of hemin in mice. The aerosol in the exposure chamber of the aerosol box remained aerosolized up to 5 minutes. A rhodamin B containing aerosol was used to show that the aerosol box gave deposition over the entire lung indicating the suitability of the model. Additionally, inhalation of hemin showed a dose dependent increase in HO-1 protein expression in the lungs. In conclusion, hemin was successfully formulated as a powder for inhalation and the inhalation model allowed controlled HO-1 expression in the lungs of mice. Future studies investigating the utility of inhaled hemin in treating disease states are warranted.

Introduction

The heme oxygenase (HO) metabolic pathway has recently received an explosion of research interest due to its potent physiological and cytoprotective properties, e.g. [1-3]. The inducible (HO-1) and the constitutively expressed (HO-2 and HO-3) forms of HO catalyze the oxidation of heme to biliverdin, carbon monoxide and iron [4]. Subsequently, biliverdin is rapidly converted to bilirubin which is a potent endogenous antioxidant [5]. All three downstream products (biliverdin/bilirubin, CO and Fe/ferritin) participate in the cellular defense. HO-1 has an integral role in the response to oxidative stress and inflammation and is induced by numerous oxidative and inflammatory stimuli [6].

Recently, it was shown that HO-1 expression in lung tissue and macrophages obtained by bronchoalveolar lavage of patients with chronic obstructive pulmonary disease (COPD) is decreased compared with healthy controls [7, 8]. COPD is a respiratory disease characterized by a chronic inflammatory response in the lungs, which is primarily caused by the toxic effects of smoking. It is tempting to hypothesize that in COPD patients an imbalance between systemic reactive oxygen species and relative antioxidant systems exists, which is possibly caused by insufficient upregulation of HO-1 [9]. One of the substances that induces HO-1 is hemin (ferriprotoporphyrin IX), the oxidized form of heme [10, 11]. Induction of the heme oxygenase pathway by inhalation of natural substrates, such as hemin, could therefore be of great benefit in diseases like COPD [12, 13]. So far, inhalation of hemin in humans has been performed only once as a control to show that the HO-1 pathway is involved in asthma [13]. In that study, nebulization of 2 ml of 10^{-4} M hemin resulted in increased levels of exhaled CO, indicating that the HO-1 pathway can be induced by inhalation of hemin. However, no direct evidence of HO-1 induction was given.

In the clinical setting, nebulization is characterized by a low deposition efficiency (typically 2-27%), and needs electricity or pressurized air for the aerosol to be created. In contrast, dry powder inhalation is characterized by higher deposition efficiency (up to 60%) and the patient delivers the driving force for the aerosol to be created [14-17]. Moreover, nebulization is accompanied by patient immobility, whereas dry powder inhalers are easy to carry and use. Furthermore, dry powder inhalation is likely to result in increased patient compliance, especially in chronic use.

The aim of this study was to formulate hemin as a powder for inhalation and to show a proof of concept for induction of heme oxygenase 1 expression in the lungs of mice by inhalation of hemin. To obtain the proof of concept we administered hemin as a powder for inhalation to mice by using a specially designed aerosol box, which is based on the Twincer® dry powder inhaler [18].

Materials

Hemin (51280) and rhodamin B (83689) were purchased from Sigma-Aldrich Chemie B.V. (Zwijndrecht, The Netherlands). Inulin, with a degree of polymerization of 23, was a generous gift of Sensus (Breda, The Netherlands). Sodium hydroxide (NaOH), hydrochloric acid (HCl) and ammonia (NH_4) were of analytical grade and

purchased from commercial suppliers. Demineralized water was used throughout the experiments.

Methods

Quantitative determination of hemin

Sample concentrations for determination of solubility and drug load were spectrophotometrically analyzed in phosphate buffered saline (PBS) with the Ultraspec 4052TDS apparatus at 385 nm (LKB, Zoetermeer, the Netherlands).

Solubility

In order to measure the maximum solubility in different alkaline solutions, 7 solutions with a pH between 11.0 and 14.0 were prepared in steps of 0.5 pH units. To these solutions excess hemin was added under constant stirring. After 30 minutes, the solutions were centrifuged at 10,000 rpm for 10 minutes. The supernatant was pipetted and diluted with a phosphate buffered solution and measured spectrophotometrically.

Powder production by spray drying

Due to an excess of sodium hydroxide in the solution to increase dissolution of hemin, the pH of that solution remains high. If such a solution is spray dried, the resultant powder causes an alkaline pH shift upon reconstitution. To avoid alkaline pH changes upon reconstitution of the spray dried powder, the pH needs adjustments during or before spray drying. Two methods were used for pH adjustment. In the first method a sodium hydroxide solution (0.7% w/v) with pH 13.5 was immediately before spray drying adjusted to pH 7.6 with hydrochloric acid. The second method consisted of spray drying an ammonia solution (1.2% w/v) with a pH of 13.5. During spray drying, ammonia evaporates and is not present in the dried powder any longer.

Four powders were spray dried. The first powder was prepared by dissolving hemin in a sodium hydroxide solution (0.7% w/v) at a pH of 13.5 to obtain a total concentration of 4.5% w/v. After pH adjustment the solution was filtered through a 1.2 µm filter. Hemin was not removed by filtration from the solution. After spray drying, this powder thus contained sodium chloride and hemin. The hemin drug load was determined to be 63% w/w.

The second powder was produced by dissolving hemin in an ammonia solution (1.2% w/v) with a pH of 13.5. After spray drying, this powder thus only contained pure hemin. This powder was used as a control in the dissolution experiments.

Finally, spray dried controls containing rhodamin B or inulin were prepared by dissolving the respective controls in a sodium hydroxide solution followed by pH adjustment before spray drying. Rhodamin B served as a control for the in vivo deposition characteristics and thus the suitability of the aerosol box; inulin served as a control for the local effect of powder inhalation with sodium chloride.

Spray drying was performed with a Büchi 190 Mini Spray Dryer (Büchi, Flawil, Switzerland) equipped with a two-fluid nozzle (0.5 mm). The solutions were sprayed at a fluid flow rate of 3.2 ml/min and an atomizing air flow rate of 800

l/h. The settings of the aspirator (14) and heater (14) resulted in temperatures at the inlet and outlet of 180 °C and 120 °C, respectively.
The obtained powders were stored in a vacuum desiccator at room temperature.

Dissolution

Dissolution experiments were carried out using an USP dissolution apparatus I (Rowa Techniek B.V., Leiderdorp, The Netherlands) at 37 °C and 100 rpm in 1000 mL phosphate buffered solution with 10 mg hemin, corrected for drug load. Samples from the dissolution vessel were spectrophotometrically analyzed at 385 nm.

Laser diffraction, scanning electron microscopy and cascade impactor analysis

Particle size distributions of the spray dried samples were measured with a Helos Compact model KA laser diffraction apparatus (100 mm lens, Fraunhofer theory) equipped with a RODOS dispersing system (Sympatec GmbH, Clausthal-Zellerfeld, Germany) operated at a dispersion pressure of 5 bar. All measurements were performed in triplicate. The particle size distribution in the exposure chamber was measured by placing the complete aerosol box in the laser beam. To avoid scattering by the Perspex walls, the light was directed through two opposing openings.
Scanning electron micrographs (SEM) were recorded with a JEOL JSM 6301-F Microscope (JEOL, Japan). The powder was dispersed on top of double-sided sticky carbon tape on metal disks and coated with 150 nm of gold/palladium in a Balzers 120B sputtering device (Balzers UNION, Liechtenstein).
The aerodynamic particle size distribution was measured with cascade impactor analysis using a multi-stage liquid impinger (Erweka, Heusenstamm, Germany) with a Twincer®-inhaler [18] attached to the induction port. A solenoid valve was used in combination with a timer to control the flow (corresponding with 4 kPa pressure difference) through the inhaler and the cascade impactor for the duration of 3 seconds. The stages of the cascade impactor were filled with 20 mL phosphate buffered saline (PBS). A total amount of ~100 mg was delivered in 10 separate doses. The concentration of the drug on the different stages was measured by UV absorbance at 385 nm; deposition was subsequently calculated as the percentage of the metered dose. The fine particle fraction (< 5 µm at the flow rate corresponding with 4 kPa across the Twincer, FPF) was calculated by interpolation of the cumulative mass plot versus effective cut-off diameter. The FPF is expressed as a percentage of the metered dose. All cascade impactor experiments were carried out in triplicate.

Aerosol box for passive pulmonary delivery of powders for inhalation

As a proof of concept of HO-1 induction by inhalation of hemin a new device was developed to allow passive, pulmonary dry powder delivery to small laboratory animals. The device, hereafter called aerosol box (figure 1), is actually an accessory for the Twincer®, an effective dry powder inhaler which delivers high dosed formulations in highly constant particle size distributions (fine particle fraction of 40-50%) independent of the pressure drop between 1 and 4 kPa [18]. The

efficient aerosol generation at low pressure drops makes the Twincer® most suitable for the aerosol box where the operator manually generates the air flow that disperses the powder. The device consists of the Twincer®, a cylinder with piston to provide a pressure drop across the inhaler and transfer of the aerosol to last part of the device, the exposure chamber in which a fan is built. Mice are attached to the exposure chamber. In total, up to twelve mice can be attached to the exposure chamber.
About 15 mg of powder, equivalent to 9.45 mg active ingredient (hemin, inulin or rhodamin B) and 5.55 mg sodium chloride, was dispersed in the aerosol box. In this study, one dose is defined as 5 minutes exposure to an aerosol that has been generated from 15 mg of drug formulation.

Animals

Female A/J mice (aged 8-10 weeks) were obtained from Harlan (Zeist, the Netherlands) and were held at the Central Animal Facility of the Groningen University. The experiments were approved by the Committee on Animal Experimentation of the University Medical Center Groningen and were performed under strict governmental law, adhering to the Principles of Laboratory Animal Care.

Experimental setup

The mice used in this study were divided into 6 groups. Group 1 consisted of one mouse that was not exposed to any aerosol and served as a control. Groups 2, 3, 4 (n=2 for each group) were exposed to 2, 4 and 6 doses of spray dried hemin, respectively. Group 5 (n=2) was exposed to 6 doses of spray dried inulin and served as control for sodium chloride that was present in the spray dried powders. Inulin, a naturally occurring oligosaccharide was chosen because oligosaccharides contribute marginally to the osmotic pressure due to their high molecular weight. Group 6 (n=2) was exposed to 6 doses of spray dried rhodamin. Group 6 was used to investigate the deposition of dispersed powder in the lungs of mice. Deposition of rhodamin was visualized in 4 µm sections of frozen lung tissue by fluorescent microscopy.

Western Blot

Protein samples were made from whole lung tissue using a sonicator (Bandelin sonopuls HD2070, Berlin, Germany). The proteins were separated on molecular weight using Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and blotted on a nitrocellulose membrane. The membrane was blocked overnight in 5% low fat milk and the proteins were detected with anti-heme oxygenase-1 (Stressgen, Victoria, Canada) followed by a peroxidase labeled goat-anti-rabbit antibody (DakoCytomation, Heverlee, Belgium). As a protein loading control the membrane was stripped using an 25mM Glycine-HCl buffer containing 1% SDS (pH:2) and stained for β-actin (Abcam loading control, Cambridge, UK) followed by a peroxidase labeled goat-anti-rabbit antibody (Dako). The bands of interest were visualized using enhanced chemiluminescence.
Band-sizes of each group were quantified using Matlab 6.5.1 (The MathWorks Inc, Natick, USA) and the Dipimage toolbox (Quantitative Imaging Group, Faculty of Applies Sciences, Delft University of Technology, the Netherlands) [19, 20].

In short, the grey-scale images were made binary with the Isodata threshold algorithm which calculates the best threshold value out of the grey value histogram of the image [21]. Data are given as the ratio between the number of pixels of the HO-1 band of interest divided by the number of pixels of the corresponding β-actin band.

Histology

HO-1 protein expression was demonstrated in 3 µm sections of paraffin embedded lung tissue with antibodies against HO-1 (Stressgen 896, San Diego, CA, United States). Presence of leukocytes was demonstrated in 4 µm sections of frozen lung tissue with the monoclonal antibody anti-CD45 (Pharmingen).

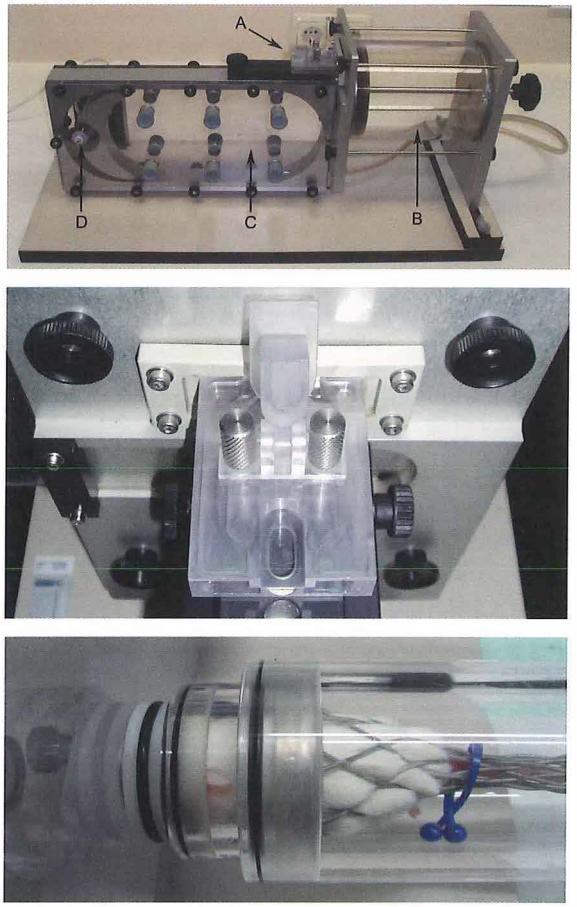

Figure 1. *(Top) The aerosol box, a utility for the Twincer® (A). By pulling the piston in the cylinder (B) an aerosol is created and can be transferred to the exposure chamber (C) to which 12 small laboratory animals can be fitted. A fan (D) creates a laminar airflow in the exposure chamber to minimize sedimentation effects. (Middle) Detail of the Twincer® attached to the aerosol box. (Bottom) Detail of mouse attached to exposure chamber.*

Results

Formulation

A series of experiments was carried out to measure the solubility of hemin at different pH levels. Hemin is practically insoluble in water (at pH ~7) and organic solvents [22]. However, under alkaline conditions hemin is soluble. In alkaline solutions, hemin (FePP-Cl) reacts with excess hydroxide to form hematin (FePP-OH). Hematin acts as an intermediate in a polymerization reaction which results in a oxy-bridged dimer and probably other polymers [22].

$FePP\text{-}Cl + OH^- \longrightarrow FePP\text{-}OH + Cl^-$
$2FePP\text{-}OH \rightarrow (FePP)_2O + H_2O$

The solubility of hemin (figure 2; solid line, primary y-axis) reached a maximum at pH 13.5. At pH>13.5 a large variation in solubility was observed which indicates the occurrence of degradation reactions, such as hydrolysis, at high pH values. Since the pH of the sodium hydroxide solution determines the amount of hemin that can be dissolved, the sodium hydroxide concentration is the main parameter that determines the efficiency of the spray drying process. The higher the sodium hydroxide concentration, the higher the hemin concentration in the solution.

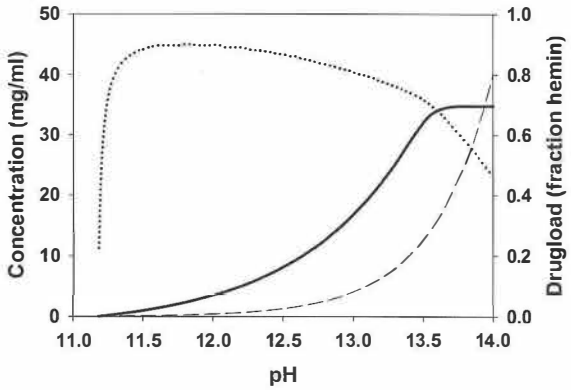

Figure 2. *Solubility of hemin (solid line, primary y-axis) as a function of pH and calculated content of hemin in spray dried powder (dotted line, secondary y-axis) as a function of pH. The calculated concentration of NaOH versus pH is represented by the dashed line (primary y-axis).(n=3)*

On the other hand, it should be realized that an increase in the sodium hydroxide concentration requires increased amounts of hydrochloric acid for neutralization. This effect will lead to a shift towards higher sodium chloride fractions in the powder, which of course reduces process efficiency in terms of drug load. Finding the most efficient process condition is a matter of balancing between the solubility of hemin, which is positively affected by the sodium hydroxide concentration, and the hemin drug load in the powder, which is negatively affected by the sodium hydroxide concentration. These effects are shown in figure 2 (dotted line, secondary y-axis).

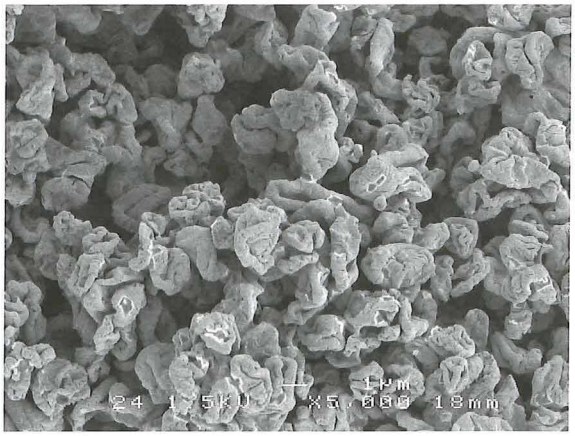

Figure 3. *Scanning electron micrograph of spray dried hemin at a magnification of 5000x.*

With increasing pH the drug load of hemin in the spray dried product initially rose up to a maximum of 90% at a pH of 11.8. At this pH the total concentration of hemin and NaOH was only 2.5 mg/ml. Spray drying of a solution with such a low concentration does not result in a high yield. Therefore the pH was increased to 13.5, which resulted in a solution with a total concentration of 45 mg/ml and a drug load of 63% after spray drying as was confirmed by chemical analysis. The spray dried hemin powder consisted of highly irregular particles (figure 3), which were in a size range that corresponded with laser diffraction (table 1).

Table 1. Particle size obtained from RODOS dispersion (5 bar) at 10, 50 and 90% cumulative undersize of the spray dried powders. All powders were spray dried from a sodium chloride solution, except pure spray dried hemin, which was spray dried from ammonia.

Formulation	x_{10} (µm)	x_{50} (µm)	x_{90} (µm)	Span*
Spray dried hemin	1.01	2.14	3.67	1.25
Spray dried hemin from ammonia	0.95	2.06	3.50	1.24
Spray dried inulin	0.92	2.29	4.66	1.64
Spray dried rhodamin B	0.87	2.02	3.42	1.26

* *Span* = $(x_{90}-x_{10})/x_{50}$

Spray dried hemin from a neutralized sodium chloride solution displayed a faster dissolution profile compared to spray dried hemin from NH_3 as shown in figure 4. After 2 minutes 50% of spray dried hemin was dissolved, compared to 20 minutes for spray dried hemin from NH_3, while the powders had approximately the same particle size (Table 1). Complete dissolution of the powder spray dried from the NH_3 solution took 110 minutes. The powder with the fastest dissolution rate was used to perform further experiments (aerosol behavior and proof of concept).

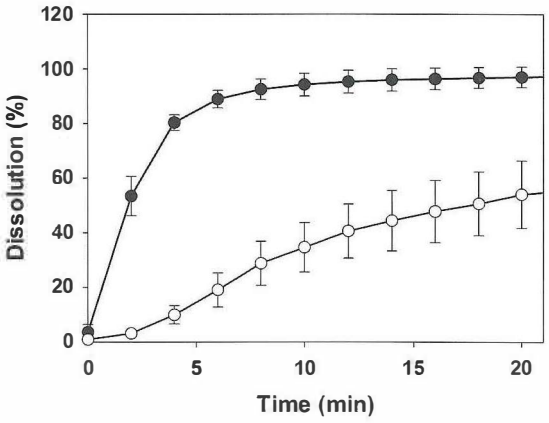

Figure 4. *Dissolution rate of spray dried hemin in the presence of NaCl (closed circle) and pure spray dried hemin from ammonia (open circle). (n=3)*

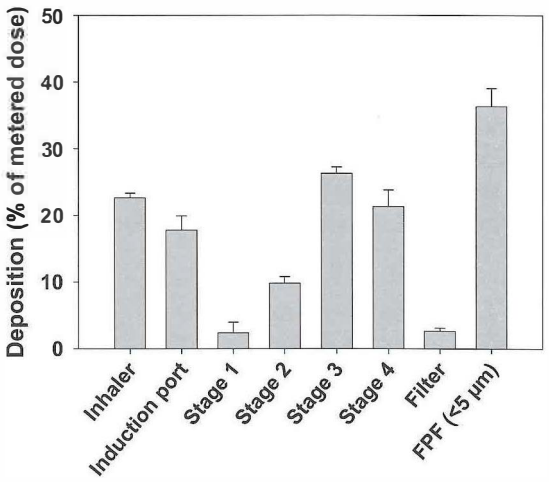

Figure 5. *Cascade impactor analysis of spray dried hemin with NaCl after dispersion by the Twincer® at 4 kPa. Fine particle fraction (FPF) represents particles <6.8 µm. Error bars represent highest and lowest value.*

In table 1 the particle size distributions of the spray dried powders are shown. The particle size distributions are comparable, only spray dried inulin had a somewhat wider size distribution.
Cascade impactor analysis (figure 5) showed a delivered dose from the Twincer at 4 kPa of approximately 77% with a fine particle fraction (<5 µm) of 36% of the metered dose.

Validation of aerosol box

After dispersion in the aerosol box (figure 6B), most particles (<90%) were between 1 and 5 µm. In the aerosol box the size distribution moved towards finer particles in time, indicating sedimentation. Larger agglomerates (5-10 µm) sediment fast and such agglomerates are no longer present after 1 minute. The change in particle size distribution will not substantially affect the inhalable fraction

in the inhaled air however, since the volume fraction of such agglomerates in the aerosol is small. Sedimentation was confirmed by a decline in the optical concentration (i.e. amount of laser light that is blocked by the aerosol). After 5 minutes the optical concentration was 0.0%, whereas the starting concentration was 1.54% on average. The amount of aerosol in the exposure chamber after 5 minutes was therefore negligible due to sedimentation. Therefore, we exposed the mice for 5 minutes per dose to the aerosol because a longer period would have no additional effect.

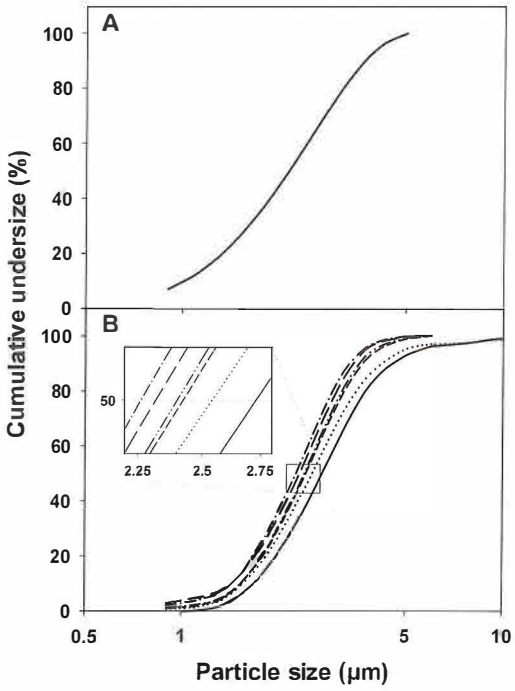

Figure 6. *(A) Geometrical particle size distributions of spray dried hemin as measured by RODOS dispersion at 5 bar and (B) in the exposure chamber of the aerosol box immediately (————), 1 minute (••••••••), 2 minutes (— — —), 3 minutes (— ••—), 4 minutes (—·——) and 5 minutes (— • — •) after dispersion by the Twincer®. (Fig.6A: n=3; Fig.6B n=4)*

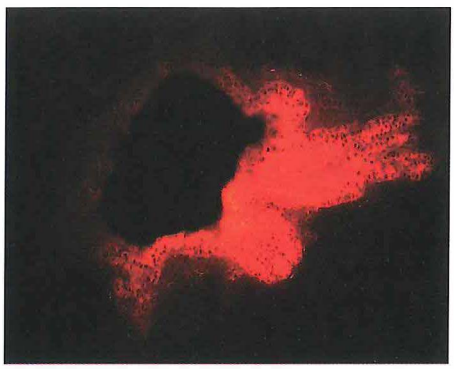

Figure 7. *Example of rhodamin fluorescence in lung tissue. The fluorescent signal (red) of rhodamine in lung tissue is shown to be present in the epithelial lining of the airways. Magnification: 20x*

To obtain an indication of the amount of drug that a mouse actually inhales, an in vitro measurement was performed by withdrawing 20 ml/min of air during 5 minutes from the exposure chamber. It was shown that this volume contained about 0.02% of each dose, an amount that consequently can be assumed as the administered dose. The assumption of 20 ml/min was on the low side, since respiratory minute volumes of mice of 45 – 50 ml/min have been described [23, 24].
In vivo deposition of inhaled powder in the lungs from the aerosol box was demonstrated by exposing mice to rhodamin B. Rhodamin B had a similar particle size distribution as spray dried hemin (Table 1). By using fluorescent microscopy, rhodamin deposition on the epithelial lining of the airways was demonstrated (figure 7). Rhodamin deposition was detected from the trachea until the large airways.

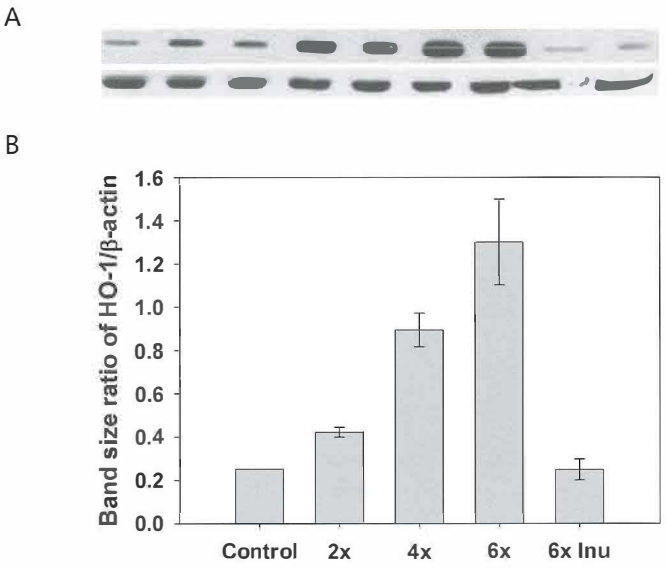

Figure 8. *(A) HO-1 protein expression. Protein bands for HO-1 (top) and β-actin (loading control, bottom) detected by Western blot analysis are shown; each band represents 1 mouse. From left to right: one control mouse that was not exposed to aerosol, 2 mice that were exposed to 2, 4 or 6 doses (2x, 4x and 6x) of spray dried hemin during 10, 20 or 30 minutes, respectively, and finally 2 mice that were exposed to 6 doses spray dried inulin (6x inu) during 30 minutes. (B) Ratio of the HO-1 and β-actin band size as obtained with Western blot for the different groups. Error bars indicate highest and lowest values.*

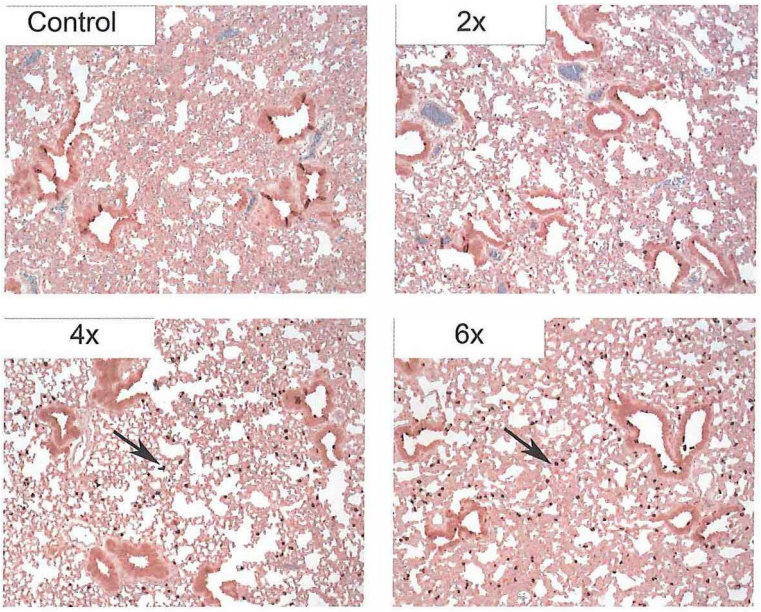

Figure 9. *A representative picture of the HO-1 expression (dark red) in lung tissue of the control and after inhalation of 2, 4 and 6 doses of spray dried hemin is shown (25x magnification). Particularly, alveolar macrophages (indicated with an arrow) show an increased HO-1 expression after hemin inhalation. Magnification: 25x*

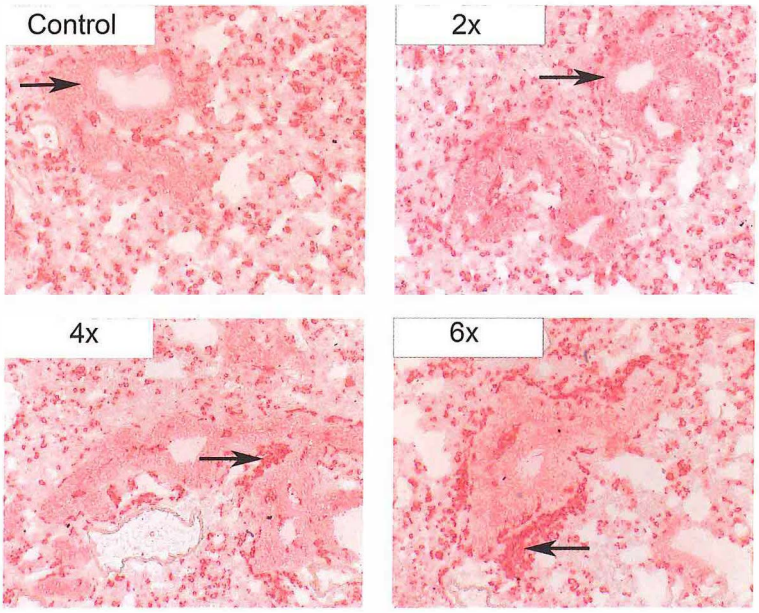

Figure 10. *Leukocytes expressed in lung tissue. A representative picture of the leukocyte expression (CD45 positive cells) in lung tissue of the control and after inhalation of 2, 4 and 6 doses of spray dried hemin is shown. Arrows in top pictures indicate airways; arrows in bottom pictures indicate areas with increased numbers of leukocytes surrounding the airways. Magnification: 50x*

Proof of concept

Different doses of spray dried hemin were administered to achieve HO-1 induction in lung tissue. Figure 8 shows a clear relationship between the dose of spray dried hemin and the level of HO-1 protein expression in lung homogenate. Furthermore, inhalation of inert powder (spray dried inulin) containing sodium chloride did not result in increased expression of HO-1.

HO-1 protein expression was found to be particularly increased in alveolar macrophages (figure 9). The airway epithelium stains faintly and shows no differences after hemin inhalation. The HO-1 expression as shown with HO-1-staining in the spray dried inulin exposed mice was comparable to control.

The inflammatory side effects of spray dried hemin inhalation were studied by histological staining of leukocytes in lung tissue. Visual observation showed increased numbers of leukocytes surrounding the airways after inhalation of 4 and 6 doses of spray dried hemin, while in the control mouse and after inhalation of 2 doses of spray dried hemin this was not visible (figure 10).

Discussion

Hemin, as shown in this study, can be formulated as a powder for inhalation by spray drying. Furthermore, spray dried hemin resulted in dose-dependent HO-1 induction after nose-only exposure of mice to the aerosol, showing that dry powder inhalation of hemin is possible and effective in inducing the HO-1 metabolic pathway.

Spray drying of hemin was performed from two solutions that had initial high pH values. An advantage of spray drying an ammonia solution that contains hemin is that ammonia evaporates during spray drying, resulting in pure hemin powder. In contrast, spray drying of the neutralized sodium hydroxide solution results in a powder that contains 37% w/w sodium chloride and 63% w/w hemin. However, the latter solution results in a powder that dissolves much more rapidly than the pure spray dried powder. This is attributed to the presence of sodium chloride in the spray dried powder which improves wetting and increases the surface over which dissolution occurs. The technique may be beneficial for other poorly soluble compounds too, especially for those compounds that have a pH-dependent solubility.

Spray drying has become one of the standard techniques for the preparation of inhalation powders [25,26]. One of the advantages of spray drying over conventional techniques such as milling is the improved control over the particle size. From both the sodium hydroxide and ammonia solution the obtained powders were in the appropriate size range for inhalation, as measured with laser diffraction and cascade impactor analysis.

Clearly, the sodium chloride in the formulation did not increase HO-1 expression as was shown by the similarity in band size of HO-1 expression of the inulin control, after 6 doses, and the control that did not receive any aerosol.

The suitability of the Twincer-based aerosol box for pulmonary administration in freely breathing mice was demonstrated in this study. The in vitro laser diffraction measurements showed that an aerosol consisting of particles suitable for inhalation is present in the aerosol box for at least 5 minutes. Moreover, the

in vivo study with rhodamin B showed that the aerosol reached all parts of the lung. It should however be realized that the individual mouse receives only a small fraction, about 0.02%, of the total dose in the Twincer. The major limitation in this setup is the relative small volume that is inhaled by mice.

One study exists where inhalation of hemin in human subjects has been investigated [13]. However, the dose in our study is much higher than the dose used in the human study. The nebulized dose in the human study was 2 ml of 10^{-4} M hemin, which is equivalent to 1.6 µg/kg for a person of 80 kg. The lowest dose in our study is estimated to be 171 µg/kg, which corresponds to at least 100 times the dose used by Horvath et al. [13].

Since there was no information available on the dose-effect relationship of hemin we exposed mice to different doses. The dose escalation in our study was thus 171, 342 and 523 µg/kg. The relatively high doses that the mice inhaled led to concerns about the toxicity of hemin since it has been reported that hemin possesses oxidative properties especially at high doses which is due to excess iron [27]. Ambiguous effects have been ascribed to hemin [28]. In moderate amounts and bound to proteins it is an essential element in various biological processes [10,29]. Large amounts of hemin can be toxic by mediating oxidative stress and inflammation, which is mainly due to release of iron [27].

The higher doses indeed led to infiltration of leukocytes into the airways. This inflammatory cell recruitment could be the result of increased production of inflammatory mediators by epithelial cells in response to toxic effects of the increasing dose of spray dried hemin. This possibility is in agreement with other in vitro and in vivo studies showing increased adhesion molecule expression and tissue infiltration of leukocytes after hemin administration to vascular cells [30,31]. On the other hand, we found that 171 µg/kg hemin did not result in increased numbers of inflammatory cells. However, inhalation of this dose of spray dried hemin was sufficient for a clear increase in HO-1 expression in lung tissue, indicating that this was a safe and effective dose of spray dried hemin.

To conclude, we presented a novel preparation of a hemin formulation by spray drying and showed proof of concept of the hemin formulation and a new pulmonary administration technique. The substance, hemin, induced the HO-1 expression in a dose dependent manner. Future studies investigating the utility of inhaled hemin in treating disease states are warranted.

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7

Chapter 8

Summary, discussion, and future perspectives

The aim of this thesis was to investigate both the early and late effects of cigarette smoke and nitrogen dioxide (NO_2) exposure and the potential dampening effects of heme oxygenase-1 (HO-1) in animal models for COPD. Additionally, we investigated the role of the specific immune response in the inflammatory response in COPD, i.e. the involvement of B cells and regulatory T cells.

Nitrogen dioxide and COPD

Short-term model

To gain more insight into the contribution of NO_2 exposure to COPD development we studied the effects of NO_2, cigarette smoke, and their combination on pulmonary inflammation and emphysema development in mice. We hypothesized that combined exposure to NO_2 and cigarette smoke would enhance both. We started with a short term model to gain more information on the early phases of inflammation by studying the effects of NO_2 exposure, in relation to cigarette smoke in this respect (chapter 2). In this study we showed that 3 hours NO_2 exposure elicits increased levels of pro-inflammatory cytokines in the lung, while short-term cigarette smoke exposure elicits increased numbers of inflammatory cells in the lung. Intriguingly, in contrast to our hypothesis, the NO_2 induced cytokine production was completely abolished when NO_2 exposure was followed by short-term cigarette smoke exposure. Possible explanations for the 'down regulatory' effects of cigarette smoke on the NO_2-induced inflammatory response include the presence of anti-inflammatory molecules in cigarette smoke, e.g. carbon monoxide (CO) [1], nitric oxide (NO) [2] and nicotine [3-5].
In order to unravel the dampening effects of cigarette smoke exposure on the NO_2 induced inflammation, we performed an additional, similar experiment with carbon monoxide (CO) instead of cigarette smoke exposure. CO exposure indeed inhibited the NO_2 induced increase in the levels of MCP-1, which was in accordance with the results found after smoke exposure. However, CO exposure had no effect on NO_2 induced increases in the levels of IL-5 and IL-6. Therefore, the 'down regulatory' effects of cigarette smoke could partly be explained by the presence of CO in cigarette smoke.
From this study we concluded that short-term NO_2 and cigarette smoke exposure lead to different inflammatory responses in mice, which can probably both contribute to the development of COPD after long term exposure. Since COPD is a chronic disease, it is likely that the initial dampening effects of cigarette smoke will have faded away after long term exposure and the combination of cigarette smoke and NO_2 may then cause increased pulmonary inflammation and enhanced development of COPD.

Long-term model

To find out whether the above is true we subsequently studied the long-term effects of NO_2 and cigarette smoke exposure and hypothesized that combined exposure to NO_2 and cigarette smoke enhances pulmonary inflammation and emphysema development in mice (chapter 3). Four weeks of NO_2 exposure resulted in increased inflammation expressed as increased numbers of goblet

cells and eosinophils, together with a small increase in the levels of IL-6 and decreased levels of IL-10. In contrast to short-term NO_2 exposure, long-term NO_2 exposure hardly affected the levels of inflammatory cytokines in this model. Four weeks of cigarette smoke exposure resulted in increased levels of TNF-α, KC, IL-6, and MCP-1 together with increased numbers of eosinophils, while there was no effect on numbers of neutrophils, macrophages, and lymphocytes. Remarkably, and contradictory to our hypothesis and the results of the short-term study, long-term NO_2 exposure dampened the cigarette smoke induced increases in the inflammatory cytokines TNF-α and KC, with a trend for MCP-1.

These dampening effects can be due to immune modulatory effects of NO_2 which have been described earlier [6-8]. NO_2 exposure can impair the function of macrophages and epithelial cells, leading to increased susceptibility to infections and development of alternatively activated macrophages. By decreasing the immune response to infections, NO_2 exposure may contribute to enhanced susceptibility to exacerbations in patients with COPD since the majority of exacerbations is associated with viral or bacterial infections [9]. This is in accordance with data showing associations between hospital admissions for COPD exacerbations and levels of air pollutants like NO_2 [10]. Furthermore, increased exacerbation rates have been suggested to cause accelerated lung function decline in patients with COPD [11]. Together, this may suggest a contributory role for air pollution in COPD development and/or progression.

Summarizing, we showed that short-term exposure to NO_2, results in a clear increase in levels of inflammatory cytokines in the lung, while after prolonged NO_2 exposure these effects fade away. Additionally, in contrast to our hypothesis, combined exposure to NO_2 and cigarette smoke exposure did not lead to an enhanced inflammatory reaction in the lungs in both models. In the short-term model this was due to anti-inflammatory effects of short-term cigarette smoke exposure. In the long-term model this was caused by the blunted inflammatory response after prolonged NO_2 exposure. These findings were unexpected and we were not able to clearly establish the contributory role for NO_2 in cigarette smoke-induced pulmonary inflammation in these mouse models. Future studies are therefore needed to further disentangle the contribution of NO_2 exposure to the inflammatory response in COPD, in particular with respect to susceptibility to infections and exacerbations. Combining NO_2 exposure with an infectious stimulus might result in a pronounced inflammatory response in the lungs, perhaps even establishing an animal model for COPD exacerbations. Longer exposure durations, as in the human situation, should also be attempted to extend our findings.

Specific immunity in COPD

Recently, the role for the specific immune response in COPD has gained increasing attention. In this thesis we hypothesized that B cells contribute to the development of COPD by means of an antigen specific immune response. Additionally, we hypothesized that Tregs are involved in the suppression of this B cell mediated immune response. In chapter 4 we demonstrated the presence of lymphoid aggregates in the airways and lung parenchyma of patients with

COPD. In addition, similar aggregates were present in our smoking mouse model. These lymphoid aggregates consisted mainly of B cells interspaced by follicular dendritic cells and surrounded by T cells. With Vh gene analysis we revealed an oligoclonal process with ongoing mutations, which suggests an antigen driven process. At present, it is unclear against which antigen(s) this B-cell reaction is directed. At least three potential sources of antigens should be considered: microbial, cigarette smoke components or derivatives, and degradation products of lung extracellular matrix.

In chapter 5 we demonstrated that Treg percentages were increased and (memory) B cells were decreased in peripheral blood of COPD patients compared to healthy individuals. Additionally, higher Treg percentages correlated with lower (memory) B cell percentages in peripheral blood, which supported our hypothesis regarding the involvement of Tregs in the B cell mediated immune response. Intriguingly, increased memory B cells, particularly class switched memory B cells were found in current smokers, regardless of the disease state. This observation supports the possibility of an antigen specific secondary immune response caused by direct smoke-elicited neo-antigens (e.g. smoke particles or matrix degradation products) in current smokers.

B cells are the key players of the adaptive immune system with respect to providing humoral immunity via the production of antibodies. The contribution of B cells in COPD could be the enhancement of innate responses by immune complex formation with lung matrix components. This process can cause lung tissue destruction and formation of degradation products of the lung matrix, which in turn may contribute to perpetuation of the inflammatory response. To what extent B cells contribute to COPD development and/or disease progression is still unclear. In this thesis we demonstrated the presence of B-cell follicles in lung tissue (chapter 4) and found decreased B cells present in peripheral blood of COPD patients (chapter 5). Together with other studies showing increased presence of B cells in the lungs of COPD patients [12,13], we propose an increased mobilization of B cells from the periphery to the lung, probably in response to increased presence of antigens in the lungs.

In chapter 6, we showed that HO-1 upregulation was associated with reduced B-cell infiltrates, but the reduced number of B-cell infiltrates was not accompanied by decreased emphysema development. This suggests that the presence of B-cell infiltrates may contribute but is not a prerequisite for emphysema development in this model. This is compatible with the results of d'Hulst et al, showing smoke induced emphysema development in scid mice, lacking functional B- and T-cells [14]. As yet, it remains unclear to what extent B cells contribute to the inflammatory response in COPD. However, since the majority of the smoking mouse models only resemble mild disease, it is conceivable that B cells might be more important in severe than in mild disease. This is supported by the data of Hogg et al who found increased numbers of airways containing B-cells and B cell follicles especially in GOLD stage III and IV [13].

Tregs are a special subset of $CD4^+$ T lymphocytes, which are important in controlling immunological tolerance and preventing auto-immune reactions by inhibiting T-cell responses [15]. Next to their effects on T cells, Tregs can also directly inhibit B-cell responses, by suppressing class switch recombination and Ig

production in B cell follicles without necessarily having to suppress the adjacent T cells [16,17]. Together with our findings on Tregs and B cells described in chapter 5, this suggests that Tregs could also be involved in the suppression of the B-cell mediated immune response in COPD. Only three other studies have assessed the presence of Tregs in COPD and they reported variable findings: a decreased percentage of Tregs and Foxp3 mRNA expression in lung tissue of emphysema patients compared to control subjects [18], an increased percentage of Tregs in BAL from COPD patients and healthy smokers compared to healthy never smokers [19], and an increased percentage of Tregs in BAL of healthy smokers compared to COPD patients and never smokers [20]. In all three studies, Tregs were also assessed in peripheral blood but no differences were found between COPD patients and healthy individuals. Our study differed with respect to co-expression of Foxp3 and showed an increased percentage of Tregs in peripheral blood of COPD patients. One possible explanation for our observations could be a decreased recruitment of Tregs from the circulation to the lung in COPD patients, leading to a decreased presence of local Tregs in the lung, which was already shown by Lee et al [18]. Notwithstanding the relevance of these findings, the functionality of Tregs is probably more important than the mere presence of these cells in COPD. Lee et al were the first to investigate Treg function in COPD, and found similar Treg function in peripheral blood of COPD patients compared to healthy individuals [18]. In chapter 5, HO-1 expression was used as a crude measure for Treg functionality, but no differences in HO-1 expression were found between COPD patients and healthy individuals using this method.

Altogether, it can be concluded that Treg frequencies may be different between COPD patients and healthy controls, between different compartments, and are also influenced by smoking status. No differences were detected in Treg functionality between COPD patients and healthy controls yet, but since Treg function can be different at the site of inflammation, depending on the local inflammatory environment [21], it is crucial to study local Tregs in the lung in COPD. Further studies aiming particularly at these local Tregs and their inflammatory environment are necessary to elucidate the exact role for Tregs in the inflammatory response in COPD.

Important questions that arise from these findings are 1- to which antigen(s) is this B cell response directed? 2- What is the potential role for these B cells in COPD development and/or disease progression? and 3- How does the local inflammatory response affect Treg presence and/or function in COPD and how does this affect its role in development or progression of disease?

To find out to which antigen(s) this B-cell response is directed, different approaches can be used. The first is a very broad screening for antibody titers in serum of COPD patients against the most likely antigens and compare these titers with healthy individuals. With this approach one is able to obtain a first clue whether antibodies against these potential antigens are present in COPD. Yet, this may seem a "searching for a needle in a haystack" acquisition. Nevertheless, a recent study already detected the presence of anti-elastin antibodies in emphysema patients, while no antibodies against collagen could be detected [18]. These results indicate an antigen-specific response against elastin, but definitely need to be reproduced in more cohorts of COPD patients and healthy individuals.

Given our earlier findings regarding decorin expression in COPD [22,23], this would be an interesting additional target to start with.
Another approach is to specifically aim at the antigen-specific B cells in the lung. In this case, one can choose to specifically isolate B cells from follicles by laser capture microscopy or isolate a certain B-cell population by flow cytometry analysis from lung homogenates of COPD patients. Next, the B-cell receptor can be cloned from these isolated B cells and expressed in a cell line. This can then be followed by a screening for antigen production in vitro.
Finally, the capacity of circulating B cells from COPD patients and healthy individuals to switch to plasma cells and produce antibodies in response to polyclonal or antigen specific (lung matrix proteins or cigarette smoke extract) stimulation can be studied in vitro using ELISPOT and ELISA.
The potential role for B cells in COPD development and/or progression can probably best be studied in animal models. Recently, the group of Kheradmand immunized mice with elastin peptides and found high anti-elastin titers together with increased numbers of activated macrophages and neutrophils, and an increase in MMP9 and MMP12 in BAL [24]. These findings indicate that the presence of anti-elastin antibodies can cause a COPD-like inflammatory reaction in the lungs, and suggest that presence of these antibodies may contribute to disease pathogenesis. Whether the presence of these antibodies ultimately leads to emphysema development in these mice is not clear yet; it could be that additional stimuli like cigarette smoke exposure are needed as well. Future animal studies combining the effects of smoking and the presence of these antibodies are probably crucial in elucidating the importance of these lung specific antibodies and the role for B cells and specific immunity in COPD pathogenesis.
The effects of the local inflammatory environment on Treg presence and function can be studied both in humans and in animal models. From resected human lung tissue Tregs can be isolated and the functionality of these cells can be studied in vitro. When sufficient lung material is available, Treg numbers and functionality can be compared between COPD patients and healthy controls. Additionally, the inhibitory capacity of these cells can be measured when they are cultured in the presence of different inflammatory mediators or cigarette smoke extract. Similar experiments can be performed with dendritic cells, which are pre-stimulated with different inflammatory stimuli, to find out whether the inflammatory status of dendritic cells influences Treg function.
In animal models, similar in vitro stimulation experiments can be performed as with human tissue, but in better defined groups. Tregs and dendritic cells obtained from acute and chronic smoke exposed mice can be compared with those of control mice e.g. with respect to proliferation and cytokine production. Furthermore, the role of Tregs in relation to B cells in the 'autoimmune' mouse model with elastin-antibody induced lung inflammation and also in similar models with other lung matrix proteins should be studied. Finally, when available, it would be very interesting to study the development of smoke induced B-cell infiltrates and emphysema in mice (temporarily) lacking functional Tregs.

HO-1 expression in COPD

Our general hypothesis stated that HO-1 is insufficiently upregulated in COPD patients leading to a higher susceptibility to the effects of cigarette smoke and oxidative stress. In chapter 6 we tested whether HO-1 modulation in our smoking mouse model could dampen the development of cigarette smoke-induced emphysema and lung inflammation.

The most important finding of this study was the protective effect of HO-1 upregulation on the development of cigarette smoke induced B-cell infiltrates, leading to reduced numbers of B-cell infiltrates in CoPP treated smoking mice. Additionally, we extended our previous observations on B-cell infiltrates (chapter 4) by the intriguing finding that the reduced number of B-cell infiltrates was accompanied by increased numbers of $CD4^+CD25^+$ Tregs in the CoPP smokers. Interestingly these findings are in accordance with earlier findings showing a direct link between Foxp3 and HO-1 expression and function of Tregs; both Foxp3 and HO-1 were shown to be expressed in Tregs and the suppressive effects of Tregs were shown to be mediated by HO-1 expression [25]. Furthermore, in a model of allergic airway inflammation, HO-1 upregulation increased Treg numbers and their suppressive capacity [26]. Moreover, in our smoking mouse model Tregs also seem to suppress B cells, which fits with existing literature [16,17], and with our findings in chapter 5.

Altogether, these findings support the concept that HO-1 expression influences Tregs and demonstrate that this mechanism can suppress tobacco smoke induced B-cell infiltrate formation. An important question that arises from these findings is how HO-1 upregulation inhibits B-cell infiltrate formation and increases Treg numbers. As mentioned before, it has been shown that the suppressive function of Tregs is mediated by HO-1 expression [25]. In order to judge whether this was also the case in our study (chapter 6), it is important to know whether the HO-1 expression in the Tregs was increased by the CoPP treatment. Unfortunately, we could not detect HO-1 expression in T cells in lung tissue, and therefore were unable to analyze this. On the other hand, contrary to the earlier findings, a recent study showed that HO-1 expression is not necessary for the development of Tregs and Treg function in mice [27]. These contradictory findings indicate that although HO-1 expression seems to be involved in Treg function, Treg function might not necessarily be dependent on the HO-1 expression in Tregs itself. It is very well conceivable that HO-1 expression of the surrounding cells, for instance dendritic cells and alveolar macrophages, regulates Treg function. In support of this theory we showed a clear increase in HO-1 expression in alveolar macrophages in the lung in chapter 6, which could have affected the Treg population in our model contributing to the suppression of smoke induced B-cell infiltrates. These alveolar macrophages are well known producers of inflammatory mediators in the innate immune response, but are also described to regulate secondary immune responses, by inhibiting responses of dendritic cells, possibly via nitric oxide, IL-10 and TNF-α production [28-30]. The exact mechanisms of this immune regulatory function of alveolar macrophages have not been elucidated. Nevertheless, it is reasonable to speculate that a high HO-1 expression of these alveolar macrophages could lead to an increased inhibitory function of these cells, possibly via direct effects of CO. This could have contributed to the increased

Treg numbers (possibly also Treg function), and the reduced numbers of B-cell infiltrates in our model.

In contrast to our hypothesis, HO-1 upregulation had no protective effects on the cigarette smoke induced increase in neutrophils, macrophages and inflammatory cytokines, and subsequent emphysema development. We had expected that upregulation would protect against the cigarette smoke induced oxidative stress in our model. Because the cells of the innate immune system i.e. epithelial cells, neutrophils and macrophages, are the first cells responding to oxidative stress in the lungs we had expected that upregulation of HO-1 particularly would have had impact on the numbers and activation state (production of inflammatory mediators) of these cells. Not finding effects on these cells and levels of inflammatory cytokines can perhaps be explained by the fact that the epithelium did not show an increased HO-1 expression after CoPP treatment. This maintains the possibility for the epithelial cells to respond to cigarette smoke by producing inflammatory mediators, which subsequently can be responsible for the increased numbers of neutrophils and macrophages. In fact, the majority of the inflammatory cytokines that were increased after smoke exposure in our study can be produced by epithelial cells in response to cigarette smoke [31-34], which supports this option. Additionally, we did not succeed to reliably measure HO-activity levels in this model, and are therefore not sure whether CoPP treatment also increased HO-activity levels next to HO-1 protein levels, as intended.

Since the CoPP treatment, which was used for the HO-1 induction in chapter 6, was stressful for the mice and did not result in increased HO-1 expression in epithelial cells, we developed a new non-invasive method for local HO-1 induction in mice (chapter 7). The purpose of this study was to formulate hemin, the natural substrate for HO-1, as a powder for inhalation and to show proof of concept of HO-1 expression in the lungs of mice by inhalation of hemin. We showed that hemin was successfully formulated as a powder for inhalation and the inhalation model allowed controlled HO-1 expression in the lungs of mice. This was proven by a dose dependent increase in HO-1 protein expression after hemin inhalation in the lungs. Along with the dose dependent increase in HO-1 expression, there was also an increased recruitment of leukocytes. This corresponds with existing literature showing that large amounts of hemin can be toxic by mediating oxidative stress and inflammation [35], and in vitro and in vivo studies showing increased adhesion molecule expression and tissue infiltration of leukocytes after hemin administration [36,37]. In accordance with the CoPP treatment, the hemin inhalation showed an increased HO-1 expression in alveolar macrophages, but also not in epithelial cells. In a follow up study, the anti-inflammatory effects of low dose hemin treatment were investigated in our model of acute NO_2-induced pulmonary inflammation in mice. Contrary to our expectations, hemin treatment did not reduce NO_2 induced pulmonary inflammation in that model and even aggravated the inflammatory effects of NO_2 for several parameters. These observations indicate that toxic and inflammatory side effects of the hemin treatment, even when using a relatively low dose, dominate the anti-inflammatory effects of the increased HO-1 expression. The fact that HO-1 expression was only increased in the macrophages and not in the epithelium may have contributed to these negative findings as well.

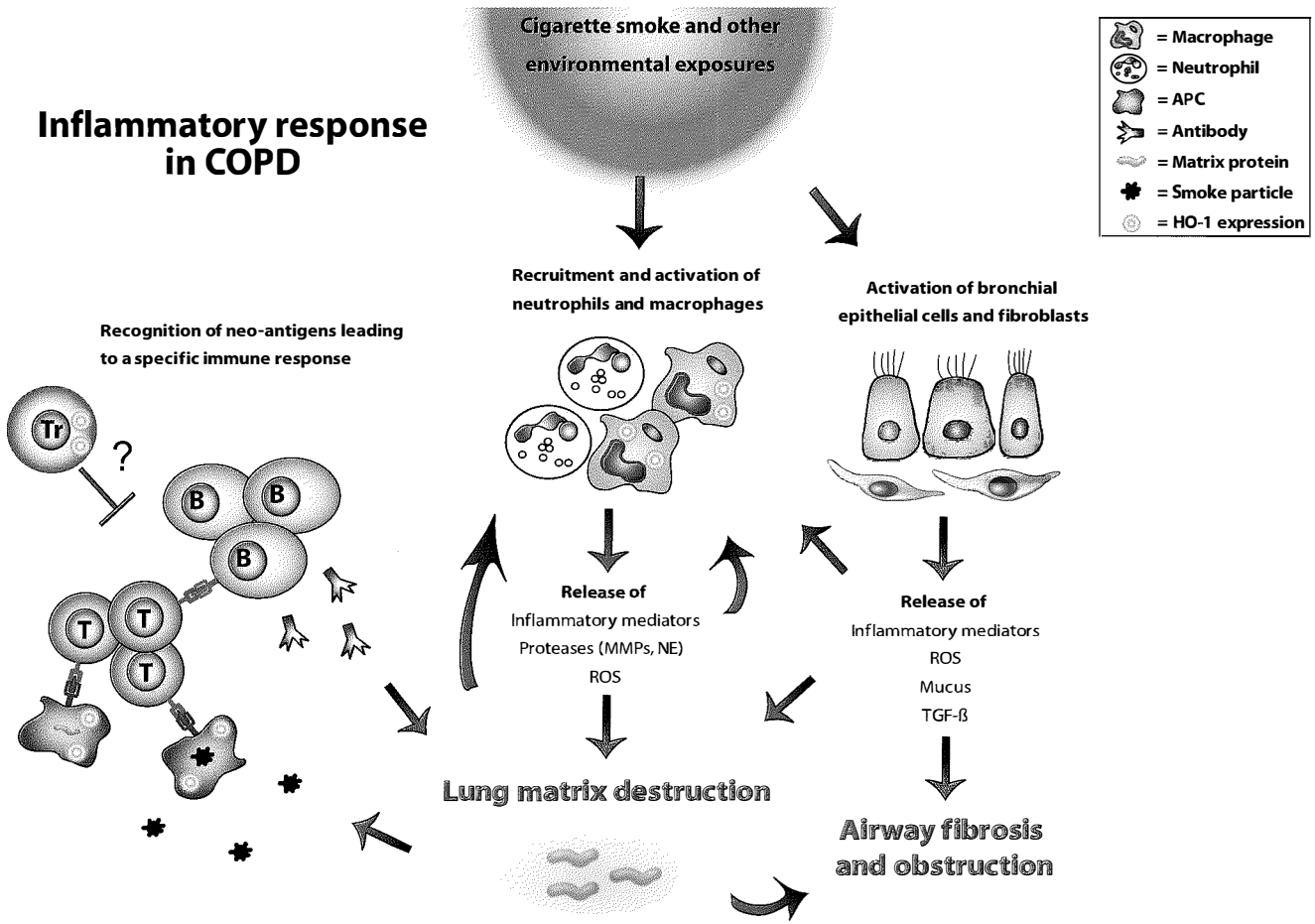
Inflammatory response in COPD
Cigarette smoke and other environmental exposures
= Macrophage
= Neutrophil
= APC
= Antibody
= Matrix protein
= Smoke particle
= HO-1 expression
Recruitment and activation of neutrophils and macrophages
Activation of bronchial epithelial cells and fibroblasts
Recognition of neo-antigens leading to a specific immune response
Tr
?
B
B
B
T
T
T
Release of
Inflammatory mediators
Proteases (MMPs, NE)
ROS
Release of
Inflammatory mediators
ROS
Mucus
TGF-ß
Lung matrix destruction
Airway fibrosis and obstruction

Figure 1: Proposed model for the inflammatory response in COPD
Smoking together with environmental exposure to toxic gases (e.g. NO_2) triggers the innate immune system (i.e. epithelial cells and macrophages) to produce inflammatory mediators (e.g. TNF-α, IL-8, GMCSF and IL-1β). This results in the recruitment and activation of inflammatory cells, which produce proteases and reactive oxidant species (ROS) causing destruction of the lung matrix. In parallel, epithelial cells produce excess mucus, and fibroblasts and epithelial cells produce TGF-β, which both contribute to development of fibrosis and airway obstruction. Lung matrix degradation proteins and/or cigarette smoke derived particles can be processed by antigen presenting cells (APCs) and presented as neo-antigens to T cells. These T cells activate antigen specific B cells, leading to a specific immune response in the lung. Normally, this specific immune response is inhibited by adequately functioning Tregs. However, in COPD patients Tregs are dysregulated, which facilitates the tissue specific antibody production by B cells. These antibodies will cause more lung matrix destruction, an increased pool of lung matrix degradation proteins, and a perpetuation of the inflammatory response in COPD. HO-1 expression can have inhibitory effects on both the innate and the specific immune response in the lungs, by its expression in alveolar macrophages, Tregs and APCs. In COPD patients HO-1 expression has shown to be decreased in alveolar macrophages, which may contribute to an increased production of inflammatory mediators. A decreased HO-1 expression in Tregs or APCs could contribute to the specific B-cell mediated immune response, possibly by affecting antigen presentation and functionality of Tregs.

From these studies it can be concluded that our model of hemin inhalation in mice is a successful method to locally induce HO-1 expression in the lung, particularly in alveolar macrophages, but due to the toxic and inflammatory side effects not a good alternative for the CoPP treatment. For future long term experiments assessing the effects of HO-1 induction on COPD development, it is probably more appropriate to use HO-1 transgenic mice, or less toxic downstream products of the HO-1 system e.g. CO or bilirubin.

Final conclusions

When combining the findings of this thesis with existing literature we propose the following hypothesis regarding the inflammatory response in COPD. A schematic representation of the inflammatory response in COPD is depicted in Figure 1.
Next to the effects on the innate immune system, smoking together with environmental exposure to other noxious gases (e.g. NO_2), elicits a B-cell mediated antigen specific immune response in the lung possibly against lung tissue matrix degradation products or smoke particles. Tregs are involved in the suppression of this B-cell mediated immune response, probably at least partially via HO-1 expression. In healthy smokers, this B-cell mediated immune response is counterbalanced by a normally functioning specific immune response, including adequate Treg function. In COPD patients an altered specific immune response has been shown; increased Tregs and decreased B cells in peripheral blood (chapter 5) together with decreased Tregs and increased B cells in the lungs [12,13,18]. This altered specific immune response in COPD might contribute to the development and/or progression of the chronic inflammatory response in the lungs of patients with COPD.

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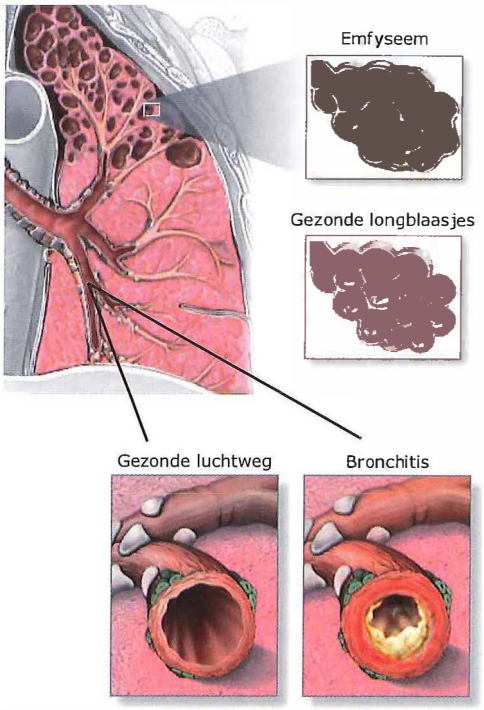
8

Chapter 9

Nederlandse samenvatting voor niet-ingewijden

Wat is COPD?

Het beschreven onderzoek in dit proefschrift gaat over de ontstekingsreactie die aanwezig is in de longen van mensen met de ziekte COPD. COPD is de Engelse afkorting voor Chronic Obstructive Pulmonary Disease en wordt in het Nederlands ook wel chronische obstructieve longziekte genoemd. Het is een chronische longziekte die gekenmerkt wordt door een combinatie van chronische bronchitis (ontsteking van de luchtwegen) en emfyseem ("rek uit de long") (Figuur 1). De chronische ontsteking van de luchtwegen zorgt voor verdikking van de luchtwegwand en overmatige slijmproductie wat resulteert in vernauwing van de luchtwegen. Bij emfyseem is er sprake van afbraak van longblaasjes, waardoor er minder capaciteit is voor zuurstof opname en er gaten vallen in het longweefsel. Door dit verlies van longweefsel wordt ook het "ophang" systeem van de luchtwegen aangetast, wat mede bijdraagt aan de vernauwing van de luchtwegen.



Figuur 1. *Schematische weergave van een long met een voorbeeld van gezonde longblaasjes in vergelijking met kapotte longblaasjes (emfyseem) en een gezonde luchtweg in vergelijking met een ontstoken en vernauwde luchtweg (bronchitis). (Aanpassing van figuren afkomstig uit de Medische Encyclopedie ADAM, http://www.nlm.nih.gov/medlineplus/encyclopedia.html)*

De belangrijkste symptomen van COPD zijn chronisch hoesten, slijm opgeven en benauwdheid bij inspanning en bij gevorderde ziekte ook in rust. De diagnose wordt grotendeels gebaseerd op de longfunctie. Hierbij wordt onder meer gekeken naar de long inhoud en de maximale hoeveelheid lucht die in 1 seconde kan worden uitgeblazen. De ernst van de ziekte wordt vervolgens op basis van deze longfunctie parameters ingedeeld in 4 stadia, volgens de zogenaamde GOLD criteria (Global Initiative for Obstructive Lung Diseases). Patiënten met

stadium 1 hebben weinig klachten en zijn zich vaak nog niet eens bewust van de ziekte. Patiënten met stadium 4 hebben een zeer beperkte longfunctie, die vaak gepaard gaat met ernstige benauwdheid bij inspanning en vaak ook in rust, chronisch hoesten en veel slijm opgeven. Bij deze groep patiënten kan een bijkomende luchtweginfectie fataal zijn. Uiteindelijk heeft COPD vroegtijdige sterfte tot gevolg en men heeft voorspeld dat COPD wereldwijd doodsoorzaak nr. 3 zal zijn in 2020.
De belangrijkste behandeling is momenteel stoppen met roken. Door te stoppen met roken kan de versnelde achteruitgang in longfunctie geremd worden en kunnen de luchtwegklachten afnemen. Helaas bestaan er nog geen medicijnen die de ziekte kunnen genezen. De huidige medicijnen bestaan uit luchtwegverwijdende en ontstekingsremmende middelen, die de klachten kunnen verlichten en verergering van de klachten kunnen voorkomen.
Hoe COPD precies ontstaat en wat de onderliggende mechanismen zijn voor de chronische ontstekingsreactie in de longen is nog grotendeels onbekend. Duidelijk is dat roken een belangrijke rol speelt in de ontwikkeling van deze ziekte. 90% van de COPD-patiënten heeft gerookt of rookt nog steeds. Echter, niet alle rokers ontwikkelen COPD; "slechts" 15-20% van alle rokers ontwikkelt de ziekte. Dit geeft aan dat naast roken ook andere factoren van belang zijn bij de ontwikkeling van COPD. Hierbij kunnen we denken aan erfelijke eigenschappen, maar ook omgevingsfactoren zoals luchtverontreiniging of meeroken.
Voor de ontwikkeling van nieuwe geneesmiddelen is verdergaand onderzoek naar het exacte ontstaansmechanisme van deze ziekte noodzakelijk. In dit proefschrift hebben we op verschillende manieren onderzoek gedaan naar mechanismen die ten grondslag liggen aan het ontstaan van COPD en de chronische ontstekingsreactie in de longen. Hiervoor werd zowel in muizen als in mensen onderzoek gedaan. De verschillende studies zullen hieronder per onderwerp worden samengevat.

Effecten van stikstofdioxide op de ontstekingsreactie in COPD

Zoals hierboven beschreven, spelen naast roken ook andere factoren zoals luchtverontreiniging een belangrijke rol bij de ontwikkeling van COPD. Stikstofdioxide, ofwel NO_2, is een belangrijke luchtverontreinigende stof die geassocieerd is met toename van luchtwegklachten in mensen en met emfyseemontwikkeling in diermodellen. Belangrijke bronnen van NO_2 uitstoot zijn de industrie, uitlaatgassen, sigarettenrook en binnenshuis stoken of koken met fossiele brandstoffen.
Uit grootschalig bevolkingsonderzoek is gebleken dat het erg lastig is om de afzonderlijke gevolgen van blootstelling aan luchtverontreiniging en sigarettenrook op het ontstaan van luchtwegklachten te onderscheiden. Dit komt doordat individuen vrijwel nooit aan één enkele verontreinigende stof worden blootgesteld. In welke mate luchtverontreiniging en/of NO_2 blootstelling bijdragen aan de ontwikkeling van COPD en mogelijk de effecten van (mee)roken kunnen verergeren is daardoor nog grotendeels onbekend.
Om de bijdrage van luchtvervuiling op de ontstekingsreactie in COPD te bestuderen hebben we de acute en chronische effecten van roken in combinatie

met NO_2 blootstelling onderzocht in een muizenmodel voor COPD (hoofdstukken 2 en 3). In deze studies hebben we muizen afzonderlijk blootgesteld aan rook en NO_2 of aan de combinatie van rook en NO_2. Op deze manier konden we zowel de afzonderlijke effecten van rook en NO_2, als de gecombineerde effecten van rook en NO_2 in kaart te brengen. Voor de blootstelling aan rook werden muizen in onze rookmachine gezet. Om rook in te ademen zitten muizen in een soort korset met de neus in de schoorsteentjes van de rookmachine. Zo ademen ze de rook via de neus in, aangezien muizen alleen via de neus ademhalen. Voor de NO_2 blootstelling werden de muizen in een afgesloten box geplaatst, waarin de NO_2 blootstelling plaatsvond.
In het acute experiment (hoofdstuk 2) werden de muizen gedurende 3 uur blootgesteld aan NO_2 en/of aan de rook van 4 sigaretten gedurende 2.5 uur. De volgende dag werden de muizen opgeofferd en werden de longen uitgenomen om de verschillende ontstekingsparameters te analyseren.
In het chronische experiment (hoofdstuk 3) werden de muizen gedurende 4 weken dagelijks blootgesteld aan NO_2 en/of sigarettenrook en na 4 weken opgeofferd.
Onze hypothese was dat de effecten van roken en NO_2 blootstelling elkaar zouden versterken en dat muizen die aan de combinatie waren blootgesteld de meeste ontsteking in de longen zouden krijgen. In het acute experiment bleek dit niet het geval te zijn. Muizen die alleen waren blootgesteld aan NO_2 hadden een sterke toename van verschillende ontstekingsmediatoren in de longen. Deze mediatoren zijn belangrijk bij de eerste afweer van de longen en zorgen ervoor dat ontstekingscellen worden aangetrokken naar de longen om daar bescherming te bieden. Muizen die alleen waren blootgesteld aan rook hadden een kleine toename van een aantal ontstekingscellen in de longen, en een minder sterke toename van de ontstekingsmediatoren dan die we vonden na NO_2 blootstelling. De meest opvallende bevinding was dat de muizen die blootgesteld waren aan de combinatie van rook en NO_2 helemaal geen toename van de ontstekingsmediatoren in de longen hadden. Het leek er dus op dat de toename die we zagen in de NO_2 muizen juist geremd werd als deze muizen ook aan rook waren blootgesteld. Deze mogelijk remmende effecten van acute rook blootstelling zijn niet nieuw en kunnen mogelijk veroorzaakt worden door de aanwezigheid van koolstofmonoxide (CO) in sigarettenrook. Van CO is bekend dat het in hoge concentraties giftig is en kan leiden tot CO vergiftiging, maar recent zijn ook ontstekingsremmende effecten beschreven van lagere CO concentraties. Om uit te zoeken of CO de remmende factor in de rook was in ons acute experiment, hebben we vervolgens muizen blootgesteld aan de combinatie van NO_2 en CO in plaats van rook. Dit experiment liet ontstekingsremmende effecten van CO zien op verschillende ontstekingsmediatoren. Slechts 1 van de ontstekingsmediatoren die na NO_2 blootstelling verhoogd was, werd geremd na CO blootstelling. Hieruit konden we concluderen dat de aanwezigheid van CO in sigarettenrook slechts voor een deel de remmende effecten van rook op de NO_2 geïnduceerde ontsteking kan verklaren.
Bij langdurige blootstelling aan sigarettenrook verwachtten we dat de acute remmende effecten van rook teniet zouden worden gedaan door de toxische effecten. Onze hypothese voor de chronische studie bleef daarom ongewijzigd en

de verwachting was dat we met chronische blootstelling juist wel de verergerde ontsteking zouden vinden na gecombineerde blootstelling aan NO_2 en rook.
Ook bij de chronische studie (4 weken) bleken de resultaten tegengesteld aan onze verwachting. Na chronische NO_2 blootstelling waren de acute effecten van NO_2 op de ontstekingsmediatoren helemaal verdwenen. Terwijl chronische rook blootstelling wel leidde tot een verhoging van bepaalde ontstekingsmediatoren. Opvallend was dat in de groep die blootgesteld werd aan de combinatie van rook en NO_2 deze effecten van rook verdwenen waren. In dit geval leek er dus een remmend effect te zijn van chronische NO_2 blootstelling. In de literatuur zijn vergelijkbare effecten van NO_2 blootstelling beschreven. Als mogelijk verklaring wordt gegeven dat NO_2 de afweerreactie van de longen aantast en dat daardoor dus geen ontstekingsmediatoren geproduceerd worden in reactie op de NO_2 blootstelling. De afwezigheid van een goede afweerreactie door de longen zou kunnen leiden tot een verhoogde gevoeligheid voor infecties. Gezien ook de aanwezigheid van NO_2 in sigarettenrook zouden deze effecten van NO_2 mogelijk een verklaring kunnen zijn voor de verhoogde gevoeligheid voor infecties in patiënten met COPD.
Samenvattend kunnen we concluderen dat de gecombineerde blootstelling aan NO_2 en rook om verschillende redenen zowel in het acute als chronische model niet resulteerde in een versterkte ontstekingsreactie in de longen. Deze bevindingen waren onverwacht en tegengesteld aan onze hypothese. Toekomstige studies zijn daarom noodzakelijk om de bijdrage van NO_2 blootstelling aan de rook geïnduceerde ontstekingsreactie in COPD verder te ontrafelen.

De specifieke immuunrespons in COPD

Het menselijke immuunsysteem kan worden onderverdeeld in een specifiek (verworven) en een aspecifiek (aangeboren) deel. De eerste afweer tegen een bepaald pathogeen (ziekteverwekker) wordt vooral gekenmerkt door een aspecifieke immuunreactie. Deze is direct werkzaam, kan reageren tegen een breed spectrum van pathogenen, maar is daardoor wel minder specifiek. Tijdens deze respons worden veel ontstekingsmediatoren geproduceerd, waardoor ontstekingscellen worden aangetrokken en/of geactiveerd voor de eerste afweer. De specifieke immuunreactie komt wat later op gang, maar is wel specifiek voor één bepaald pathogeen, en zal uiteindelijk zorgen voor een meer gerichte en sterke afweer. Bovendien zorgt de specifieke immuunreactie vaak voor een langdurige bescherming door de productie van antilichamen en de vorming van geheugen cellen. In sommige gevallen kan het specifieke immuunsysteem ook reageren tegen lichaamseigen antigenen, wat kan leiden tot een auto-immuunziekte.
Lange tijd werd de chronische ontstekingsreactie in de longen van COPD-patiënten voornamelijk toegeschreven aan een verhoogde aanwezigheid en/of activiteit van cellen en mediatoren van het aspecifieke immuunsysteem. Recente studies hebben echter aangetoond dat ook cellen van het specifieke immuunsysteem mogelijk een belangrijke rol spelen in deze chronische ontstekingsreactie.
Om meer inzicht te krijgen in de daadwerkelijke bijdrage van deze specifieke immuunreactie in de ontstekingsreactie in COPD hebben we de aanwezigheid van 2 typen cellen van het specifieke immuunsysteem in COPD bestudeerd, te

weten B-cellen en T-cellen. B-cellen zijn de cellen die de antilichamen produceren die belangrijk zijn bij de specifieke afweer tegen pathogenen. Uit eerdere studies is gebleken dat er een toename is van B-cellen en B-cel-infiltraten (clusters van B-cellen) in de longen van COPD-patiënten. Echter, momenteel is de rol en dus de relevantie van deze B-cellen in COPD nog onduidelijk.
T-cellen kunnen worden onderverdeeld in verschillende soorten waaronder helper T-cellen en regulerende T-cellen. Helper T-cellen zijn actief betrokken en noodzakelijk voor een sterke antilichaam respons door B-cellen. Regulerende T-cellen vormen een aparte subset van T-cellen, die immuunreacties kunnen remmen. Afwezigheid en/of disfunctie van deze regulerende T-cellen kan bijdragen aan het ontstaan van auto-immuunziekten of chronische ontstekingsreacties. Gedacht wordt daarom dat deze regulerende T-cellen mogelijk ook betrokken kunnen zijn bij de chronische ontstekingsreactie in COPD.
Onze hypothese was dat er een specifieke immuunreactie is van B-cellen in de longen van COPD-patiënten, die belangrijk is bij de ontwikkeling en verergering van de ziekte. Daarnaast denken we dat regulerende T-cellen belangrijk zijn bij het remmen van deze B-cel gemedieerde immuunreactie. Mogelijk draagt een verminderde aanwezigheid en/of functie van deze regulerende T-cellen bij aan de chronische ontstekingsreactie in COPD.
Om deze hypothesen te testen hebben we de aanwezigheid van B-cel-infiltraten en de specifieke eigenschappen van deze B cel infiltraten bestudeerd in longweefsel van COPD-patiënten en in muizen uit ons rokende muizenmodel (hoofdstuk 4). Daarnaast hebben we gekeken naar de aanwezigheid van regulerende T-cellen, B-cellen en geheugen B-cellen in bloed van COPD-patiënten en gezonde vrijwilligers (hoofdstuk 5).
In hoofdstuk 4 hebben we de aanwezigheid van B-cel-infiltraten aangetoond in longen van COPD-patiënten en in longen van muizen die langere tijd aan rook waren blootgesteld. Vervolgens hebben we laten zien dat deze B-cel-infiltraten voornamelijk bestaan uit B-cellen, maar omringd zijn door helper T-cellen. Door met genetische analyse te kijken naar de antilichamen die geproduceerd worden door verschillende B-cellen uit hetzelfde infiltraat hebben we aangetoond dat deze cellen nagenoeg allemaal hetzelfde antilichaam maken en waarschijnlijk specifiek tegen 1 antigeen reageren. Waartegen deze B-cellen in de long reageren is op het moment nog onduidelijk en wordt verder onderzocht. Het zou kunnen zijn, dat de B-cellen reageren tegen producten (antigenen) die aanwezig zijn in sigarettenrook en neerslaan in het longweefsel. Een andere mogelijkheid is, dat er door de sigarettenrook geïnduceerde ontstekingsreactie in de longen afbraak van het longweefsel plaatsvindt. Van deze afbraakproducten is bekend dat ze ontstekingsreacties op kunnen wekken. Mogelijk kunnen deze afbraakproducten door het immuunsysteem als lichaamsvreemd worden herkend en kunnen B-cellen vervolgens antilichamen gaan maken tegen deze afbraakproducten. Deze antilichamen kunnen dan een ongewenste immuunreactie veroorzaken die gericht is tegen het eigen longweefsel, met daardoor nog meer afbraak van het longweefsel als gevolg, leidend tot een auto-immuunreactie. De antigenen waartegen de B-cellen reageren kunnen theoretisch ook van bacteriën of virussen afkomstig zijn.

In hoofdstuk 5 hebben we aanwezigheid van hogere percentages regulerende T-cellen en lagere percentages B-cellen aangetoond in bloed van COPD-patiënten in vergelijking met gezonde vrijwilligers. Daarnaast vonden we dat hogere percentages regulerende T-cellen gecorreleerd waren met lagere percentages B-cellen. Deze bevindingen waren een beetje onverwacht, gezien de reeds bekende toename van B-cellen in longweefsel van COPD-patiënten en de hypothese over de afwezigheid en/of disfunctie van regulerende T-cellen in COPD. Echter voor beide cel typen geldt dat we alleen naar de aanwezigheid in bloed hebben gekeken en dat daar waarschijnlijk andere factoren van invloed zijn op de aanwezigheid van deze cellen dan in de long. Het is daarom lastig om deze bevindingen te vergelijken met bevindingen die gedaan zijn in de long. Niettemin suggereren deze resultaten dat er een veranderde specifieke immuunreactie is in patiënten met COPD.
Naast regulerende T-cellen en B-cellen hebben we ook gekeken naar de aanwezigheid van geheugen B-cellen. Geheugen B-cellen worden gevormd na een specifieke reactie van B-cellen tegen een antigeen en zorgen voor een snelle immuunreactie bij herhaalde blootstelling aan hetzelfde antigeen. Opvallend was dat we een toegenomen percentage vonden van deze geheugen B-cellen in bloed van rokende COPD-patiënten en gezonde vrijwilligers. Verhoging van geheugen B-cellen in bloed van actieve rokers suggereert dat roken zorgt voor een continue antigeen prikkel, die een specifieke immuunreactie opwekt wat deze toename veroorzaakt in het bloed.
Wat betreft de rol van het specifieke immuun systeem in COPD kunnen we tot nu toe concluderen dat er aanwijzigen zijn voor een veranderde specifieke immuun respons in COPD-patiënten; aanwezigheid van B-cel-infiltraten in de long en veranderde verhouding tussen B-cellen en regulerende T-cellen in bloed. Daarnaast lijkt roken een antigeen specifieke immuun respons op te wekken, maar tot op heden is nog onduidelijk waartegen deze respons precies gericht is.

HO-1 expressie in COPD

Een intrigerend eiwit wat mogelijk een rol kan spelen in de ontwikkeling van COPD is het `beschermende' enzym haem oxygenase-1 (HO-1). HO-1 is het enzym dat de afbraak van hemoglobine tot bilirubine, vrij ijzer en koolstof monoxide (CO) reguleert in het lichaam. HO-1 is een stressrespons eiwit wat voornamelijk geïnduceerd wordt door oxidatieve stress. Een belangrijke bron van oxidatieve stress is roken. Van zowel bilirubine, vrij ijzer als CO zijn beschermende eigenschappen beschreven en via deze reactieproducten kan inductie van HO-1 mogelijk bescherming bieden tegen de schadelijke effecten van rook.
Eerder onderzoek heeft een verminderde aanwezigheid aangetoond van HO-1 in ontstekingscellen in de longen van COPD-patiënten. Daarnaast is er een genetische variant gevonden in het HO-1-gen wat geassocieerd is met emfyseemontwikkeling.
Onze hypothese was dat in COPD-patiënten het HO-1-eiwit onvoldoende is opgereguleerd waardoor deze patiënten gevoeliger zijn voor de schadelijke effecten van sigarettenrook en oxidatieve stress.

Om meer inzicht te krijgen in de mogelijke rol van HO-1 in COPD hebben we in hoofdstuk 6 onderzocht of verhoging of verlaging van de HO-1-expressie de ontwikkeling van emfyseem en de ontstekingsreactie in de longen kon beïnvloeden in een rokend muizenmodel.
Hiertoe hebben we muizen gedurende 20 weken blootgesteld aan sigarettenrook en door het toedienen van een HO-1 verhogende of remmende stof geprobeerd de HO-1-expressie te verhogen of verlagen.
We zijn erin geslaagd met de toediening van de HO-1 verhogende stof de eiwitexpressie van HO-1 in de long te verhogen. De verlaging van de HO-1-expressie was echter niet overduidelijk. Daarnaast bleek ook de rookexpositie een hoge HO-1-expressie te veroorzaken, wat overeenkomt met de bovengenoemde stressrespons van HO-1 na een oxidatieve prikkel (= roken).
In tegenstelling tot onze verwachting had de verhoogde HO-1-expressie geen beschermende effecten op de toename in emfyseem en ontstekingsmediatoren in de longen van de muizen die aan rook waren blootgesteld. Opvallend was wel dat de toename van HO-1-expressie zorgde voor een verminderde aanwezigheid van B-cel-infiltraten in de longen van rokende muizen. Een mogelijke verklaring voor deze verminderde aanwezigheid van B-cel-infiltraten is de toegenomen aanwezigheid van de regulerende T-cellen in deze muizen. Dit komt overeen met de bestaande literatuur waarin beschreven is dat regulerende T-cellen B-cel-responsen kunnen remmen. En waarin een relatie is beschreven tussen HO-1-expressie en regulerende T-cellen, die laat zien dat de functie van regulerende T-cellen afhankelijk is van de HO-1-expressie.
Onze resultaten ondersteunen het concept dat HO-1-expressie belangrijk is bij de functie en/of aanwezigheid van regulerende T-cellen, zoals beschreven in de literatuur. Daarnaast suggereren ze dat regulerende T-cellen betrokken zijn bij het remmen van de rookgemedieerde B-cel-respons.
Een nadeel van de gebruikte methode van de HO-1-verhoging was dat de muizen wekelijks geïnjecteerd moesten worden met de HO-1 verhogende stof wat leidde tot huidirritatie op de plaats van injectie. Om voor vervolgstudies de methode van HO-1-verhoging te verfijnen hebben we een minder ingrijpende methode ontwikkeld om de HO-1-expressie te verhogen in de longen van muizen, welke beschreven is in hoofdstuk 7. In tegenstelling tot de methode met de injectie van chemische stoffen, wordt bij deze nieuwe methode het natuurlijke substraat van HO-1, hemine, in poedervorm door muizen geïnhaleerd. Voor de ontwikkeling van deze methode hebben we hemine geformuleerd als een droogpoeder met de juiste eigenschappen om een goede depositie in de longen te kunnen bewerkstelligen. Om deze formulering te testen hebben we een kleine groep muizen verschillende doses van het heminepoeder laten inhaleren. Dit experiment liet een mooie dosisresponsrelatie zien tussen de hoeveelheid geïnhaleerd poeder en de HO-1-expressie in de longen. Dus hoe meer hemine er geïnhaleerd was, hoe hoger de HO-1-expressie was in de longen. Daarnaast bleek er ook een relatie te zijn tussen de hoeveelheid geïnhaleerd hemine en de aanwezigheid van ontstekingscellen in de longen van de muizen. Een hoge dosis hemine resulteerde in een zeer hoge HO-1-expressie, maar ook in de aanwezigheid van ontstekingscellen, een negatieve bijwerking van een te hoge hemine-depositie. Bij een lage dosis hemine was dit effect op ontstekingscellen niet waarneembaar

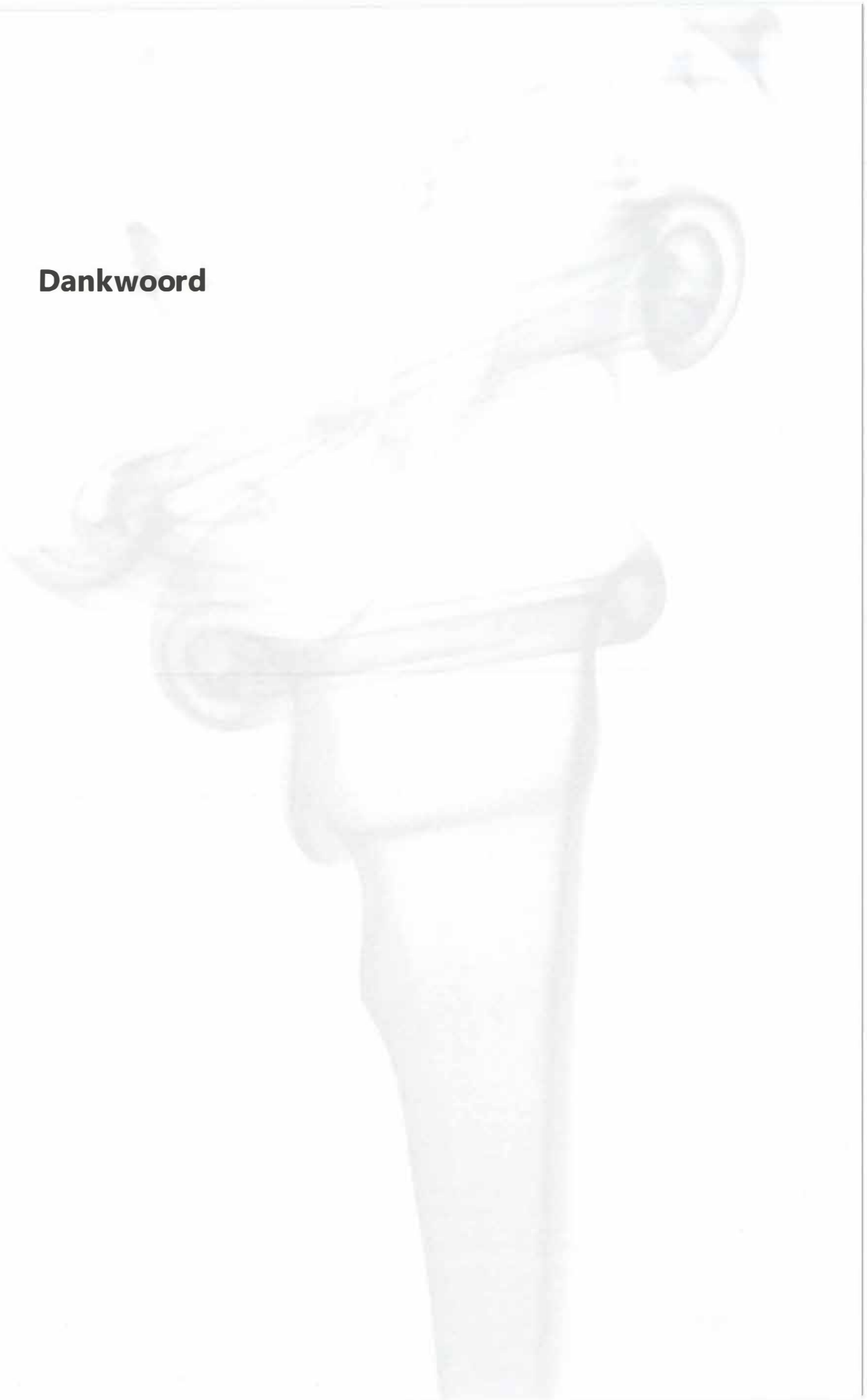
en was er nog steeds een duidelijke toename van de HO-1-expressie. De lage dosis hemine is daarom het meest geschikt voor toepassing in vervolgstudies.

Algemene conclusie van dit proefschrift

Met de verschillende studies die beschreven zijn in dit proefschrift hebben we getracht meer inzicht te krijgen in het ontstaansmechanisme van COPD en de mechanismen die ten grondslag liggen aan de chronische ontstekingsreactie in de longen.
Als we onze bevindingen combineren met de bestaande literatuur komen we tot de volgende hypothese over de chronische ontstekingsreactie in COPD:

- Roken en/of expositie aan luchtverontreiniging en meeroken veroorzaakt eerst een ontstekingsreactie in de longen, die vooral gekenmerkt is door ontstekingscellen en ontstekingsmediatoren van het aspecifieke immuunsysteem (hoofdstuk 2).
- Naarmate de ontstekingsreactie langer duurt en er schade ontstaat van het longweefsel, gaan ook cellen van het specifieke immuunsysteem, in het bijzonder B-cellen, een rol spelen in de ontstekingsreactie (hoofdstuk 4,6).
- Deze B-cel reactie is mogelijk gericht tegen afbraakproducten van longweefsel of producten van sigarettenrook die neergeslagen zijn in longweefsel.
- Regulerende T-cellen zijn belangrijk bij het controleren van deze B-cel-respons, mogelijk via de expressie van HO-1 (hoofdstuk 6).
- In 'gezonde' rokers wordt deze specifieke B-cel-respons tegengewerkt door een goed werkend specifiek immuunsysteem met goed functionerende regulerende T-cellen.
- In COPD-patiënten is sprake van een veranderd specifiek immuunsysteem; toename van regulerende T-cellen en afname van B-cellen in bloed (hoofdstuk 5) en het omgekeerde in longweefsel (literatuur). Dit veranderde specifieke immuunsysteem draagt mogelijk bij aan de ontwikkeling en/of de verergering van de chronische ontstekingsreactie in COPD.

Dankwoord



Wat mij betreft is promoveren een echte teamsport, waarbij een goed team om je heen een eerste vereiste is.
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